

**THE RELATIONSHIP BETWEEN HIGH GRAVITY
BREWING, KEY PERFORMANCE INDICATORS AND
YEAST OSMOTIC STRESS RESPONSE**

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ABSTRACT

High Gravity (HG) and Very High Gravity (VHG) fermentations are increasingly attractive within the brewing industry as a means of energy-saving and to optimise process efficiency. However, the use of highly concentrated worts is concomitant with a number of biological stress factors and in particular elevated osmotic pressure, which can impact on yeast quality and fermentation performance. In order to eliminate or reduce such negative effects, yeast cells often respond to their environment by adapting their central carbon metabolism and by making osmotic adjustments. The aim of this research was to investigate the impact of wort gravity on carbon flux, key performance indicators and to examine the effect of external osmolality (as a measure of osmotic pressure) on cell physiology.

The fermentation performance of lager and ale brewing yeasts in standard (13 °P), HG (18 °P) and VHG (24 °P) worts was assessed with respect to the uptake of wort sugars, and the production of key carbon metabolites. Estimation of carbon partitioning revealed that products including trehalose, glycogen, higher alcohols and esters had only minor effects on carbon distribution, whereas the production of yeast biomass acted as a major trade-off with ethanol production. Moreover, parallel analysis of the fermentation environment indicated that osmolality increased during fermentations, particularly at high gravities, with the largest contribution directly related to ethanol production. Consequently, yeast cells were subjected to a series of increasing osmolality levels induced by sorbitol, designed to replicate high gravity conditions. These conditions were shown to have a negative impact on yeast viability and vitality, although cell genome integrity was unaffected. In addition, cells responded to osmotic pressure by modifying membrane components leading to a change in fluidity, and by promoting glycerol production. It is anticipated that the data presented here will provide a greater understanding of the response of yeast to HG and VHG conditions, potentially leading to process optimisation in the future.

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LIST OF ABBREVIATIONS

bp	Base pairs
cm	Centimetre
°C	Degree centigrade
g	Gram
Kb	Kilobase
L	Litre
m/m	Mass/mass concentration
w/v	Mass/volume concentration
μL	Microliter
μM	Micromolar
mL	Millilitre
mm	Millimetre
mM	Millimolar
mOsm	Milliosmole
min	Minute
M	Molar concentration
ng	Nanogram
nm	Nanometre
%	Percent
Psi	Pounds per square inch
rpm	Revolutions per minute
sec	Second
V	Volt
v/v	Volume/volume concentration

CHAPTER 1: INTRODUCTION

1.1 The brewing process

Brewing is one of the world's oldest biotechnological processes and beer production currently ranks as one of the largest in the beverage sector. In order to produce beers (which can differ widely in appearance and flavour), a variety of raw materials are required, including grains, water, hops, yeast and often sugar adjuncts (Boulton & Quain, 2001). The brewing process (Figure 1.1) can be broadly divided into wort production (Section 1.1.1), fermentation (Section 1.1.2) and post-fermentation (Section 1.1.3).

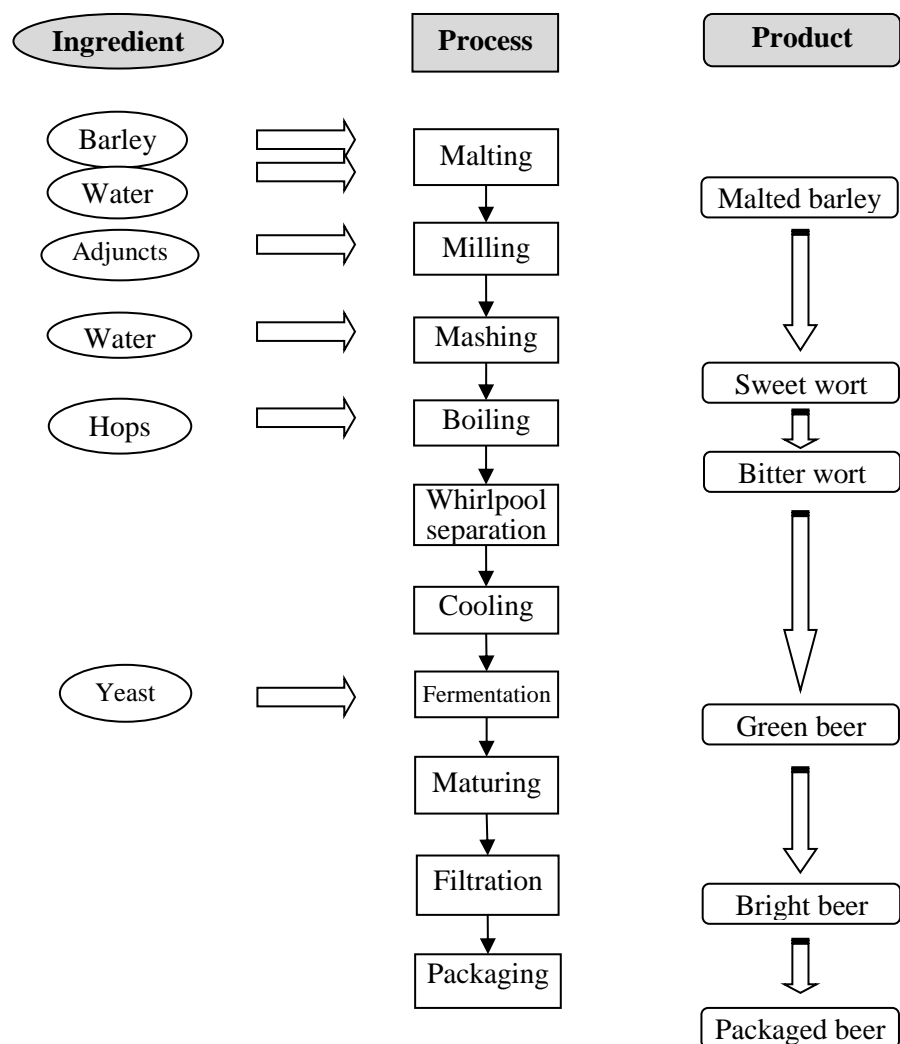


Figure 1.1 Outline of brewing process

1.1.1 Wort production

1.1.1.1 Malting

A form of fermentable sugars is essential for alcoholic fermentation. Unlike the crushed grape used in wine production, beer obtains its fermentable sugars from selected cereal grains, typically barley (Briggs, *et al.*, 2009). Malting is the process whereby the grains are converted to malt through controlled germination (Briggs, *et al.*, 2009). The grains are initially steeping in water, which results in an increase in water content, encouraging germination (Lewis & Young, 1995, Bamforth, 1998, Boulton & Quain, 2001). Once the designated point is reached, growth is terminated by drying in a kiln. By varying the temperature and times of kilning, malt can be made with various flavours and colour, which are key determinants in dictating the flavour of beer (Gibson, 1989, Briggs, *et al.*, 2009, Li & Maurice, 2013).

1.1.1.2 Milling

Milling is the process designed to convert the malt into coarse flour, termed ‘grist’. Contents of the malt endosperm are released from the husk and the mixture is reduced to a specific particle size, desired for efficient operation during the mashing procedure (Section 1.1.1.3) (Briggs, *et al.*, 1981, Lewis & Young, 1995).

1.1.1.3 Mashing

Mashing is the stage of the process where the grist is mixed with brewing water (liquor), along with an appropriate combination of pH and temperature, in a vessel called the ‘mash tun’. The extract produced during mashing is known as ‘sweet wort’ (Figure 1.1), containing a multitude of soluble components including carbohydrates, amino acids, ions and short peptides (Stewart, *et al.*, 1983). The sweet wort is separated from the spent grains by either

passing it through a mash filter bed, or via a separate process known as lautering (Lewis & Young, 1995).

1.1.1.4 Wort boiling

The sweet wort is boiled in a copper or boiling kettle, where hops, and potentially liquid adjuncts (Section 1.3.5), are added. This serves a number of purposes, including sterilization of the wort, termination of enzyme reactions, iso-merisation of hop alpha-acids, and removal of undesirable volatiles and haze-causing substances. Once boiling is finished, the hopped wort is separated from the remaining solids (termed ‘trub’) in a whirlpool tank, cooled, and pumped into a fermentation vessel where yeast is added to initiate fermentation (Bamforth, 1998, Boulton & Quain, 2001).

1.1.2 Fermentation and yeast handling

The various yeast management activities that occur within the brewery are collectively known as yeast handling (O’Connor-Cox, 1997, Kennedy, 2000, Lodolo, *et al.*, 2008). These processes are found either side of fermentation and include yeast propagation and pitching, as well as collection and storage.

1.1.2.1 Yeast storage and propagation

Prior to propagation, yeast cultures are obtained from long term storage and used to produce working cultures maintained on solid agar plates or slopes. The principle method for long term storage of cultures is to cryogenically freeze yeast cells in liquid nitrogen at -196 °C (Wellman & Stewart, 1973). Frozen samples are then either stored in liquid nitrogen, or in cryovials at -70 °C (Hulse, *et al.*, 2000). Moreover, for security purposes, yeast strains are often stored by a third-party, such as NCYC (National Collection of Yeast Cultures), CARA technology, or the Siebel Institute of Technology.

Propagation itself is a stepwise aerobic process which involves the cultivation of yeast (from the working culture) in liquid media, using incremental changes in volume (Anderson, 1994). This is performed in order to generate sufficient biomass (and the desired viable yeast cell count) to pitch full scale fermentation vessels (Quain, 1995, Kennedy, 2000, Lodolo, *et al.*, 2008). The propagation process itself can be divided into two main steps: laboratory and brewery process. During the 'laboratory stage', the yeast is cultivated in a progressively increasing volume of medium (in general, a scale-up volume of about 1:10 is applied) until sufficient yeast is available to be transferred to the larger scale 'brewery propagation'. In the laboratory, growth conditions are carefully controlled and incubation temperatures typically in the range of 25-27 °C. The yeast cells are then transferred to the brewery where the second phase of propagation occurs. In the brewery, cultivation occurs in a dedicated propagation vessel, where the primary aim is again to provide favourable conditions for yeast growth (Lodolo, *et al.*, 2008). Typically yeast is grown in sterile wort, with sufficient oxygen to encourage aerobic growth and achieve the desired biomass production (Cahill, *et al.*, 2000).

Some breweries prefer to use dried yeast for easier storage and transport, a form which is particularly suitable for small-scale craft brewers (Quain, 2006, Van Zandycke, *et al.*, 2009). However, irrespective of the form of the yeast inoculum, once the biomass and the desired cell count has been obtained, the yeast cells are added to hopped wort within a fermentation vessel in a process known as pitching.

1.1.2.2 Yeast pitching

Pitching refers to the inoculation of wort with a known and constant number of viable yeast cells. Sterile conditions are required to prevent contamination from bacteria and wild yeast. The pitching rate is controlled to ensure desired fermentation performance based on yeast viability and vitality, wort composition, specific gravity, temperature and dissolved oxygen (DO) (O'Connor-Cox & Ingledew, 1989, Stewart, 1996, Briggs, *et al.*, 2009). Although each

product typically has its own specific pitching rate, based on the criteria above and the properties of the individual yeast strain employed, many brewers typically calculate the number of cells to introduce to a fermentation based on a 1×10^6 viable cells/mL wort per degree Plato (Stewart, 2009).

1.1.2.3 Serial repitching

Serial repitching is a yeast recycling practice which is common within the brewing industry (Lawrence, *et al.*, 2012, Vieira, *et al.*, 2012, Miller, *et al.*, 2013). The practice of serial repitching commences with the pitching of a fresh yeast culture (Section 1.1.2.2), and involves fermentation (Section 1.1.2.4), cropping (recovery) of the yeast at the end of fermentation (Section 1.1.2.5), the storage of the cropped yeast (Section 1.1.2.6), and the re-use of the culture in a subsequent fermentation. A typical regime in a modern brewery introduces propagated yeast every 5-20 generations (Briggs, *et al.*, 2009), which ensures that a healthy culture, of known quality and guaranteed identity, is employed. Continual serial repitching of yeast may be associated with gradual deterioration in yeast condition, which can result in a decline in fermentation performance, leading to reduced utilization of nitrogenous compounds (Miller, *et al.*, 2013), altered sugar assimilation rates (Donnelly & Hurley, 1996) and genetic instability (Jenkins, *et al.*, 2001, Jenkins, *et al.*, 2003, Powell & Diacetis, 2007, Lawrence, *et al.*, 2012, Powell & Nguyen, 2012). In addition, the number of times a culture can be reused is largely influenced by process parameters. Particularly, if wort gravity is elevated (Section 1.3.6), the number of cycles that can be employed can be reduced (Stewart, 2009).

1.1.2.4 Fermentation

Brewery fermentations are conducted using batch-type systems, where fermentation describes the cumulative effect of yeast growth on wort, resulting in the spent growth medium: beer (Lodolo, *et al.*, 2008). Cylindroconical vessels (CCV's) are the most commonly used high-

capacity fermenters worldwide, arguably due to the use of stainless steel, an ideal construction material. Traditional fermentation temperatures for lager yeast (Section 1.2.1) range from between 8 °C and 15 °C whereas those using ale yeast (Section 1.2.1) are conducted at higher temperatures (about 20 °C) (Boulton & Quain, 2001). Oxygen is usually supplied either by wort ‘aeration’ (using sterile air) or ‘oxygenation’ (using sterile oxygen) prior to pitching (Verbelen, *et al.*, 2009a, Verbelen, *et al.*, 2009b), and this is used by cells for lipid synthesis, which is in turn essential for cell division to occur. During fermentation, yeast cells take up wort nutrients (Section 1.3), initially multiplying between 2-4 times (Powell C, personal communication) and ultimately forming ethanol, carbon dioxide and to a lesser extent other metabolites which contribute to the character of beer (Section 1.4.5). Towards the end of fermentation, the yeast cells are separated from the medium by a naturally occurring phenomena termed flocculation (Stratford & Keenan, 1987, Stratford, 1992), and sediment towards the cone located at the bottom of the fermentation vessel, where they are removed (cropped) (Section 1.1.2.5) and potentially repitched (Section 1.1.2.3). The product of fermentation is termed ‘green beer’ (Figure 1.1), which in most cases is subjected to post-fermentation processing (Section 1.1.3).

1.1.2.5 Yeast cropping

Cropping describes the removal of yeast biomass at the end of fermentation and is followed either by yeast storage (Section 1.1.2.1) and recycling of the biomass into a subsequent fermentation (Section 1.1.2.4), or by yeast disposal. Yeast should ideally be cropped as soon as possible once a fermentation has ended and appropriate hygiene standards are applied to prevent contamination (O’Connor-Cox, 1997, Boulton & Quain, 2001). This is largely because subsequent fermentation performance can be influenced by the quality of the yeast after storage (Boulton & Quain, 2001, Quain, *et al.*, 2001). Many breweries only crop yeast present in the middle portion of the cone because the bottom sediment is often enriched with

dead cells and trub, while the top portion can comprise less flocculent cells, which may affect beer quality and process efficiency in subsequent fermentations (Stewart, 1996, O'Connor-Cox, 1997). The existence of environment- and yeast-related gradients within the cone has also been identified, potentially resulting in heterogeneous sub-populations that may exhibit inconsistent physiology and fermentation performance on pitching (Barker & Smart, 1996, Deans, *et al.*, 1997, Hodgson, *et al.*, 1999, Powell, *et al.*, 2002, Powell, *et al.*, 2003, Powell, *et al.*, 2004).

1.1.2.6 Yeast storage

If cropped yeast is to be re-used (Section 1.1.2.3), temporary storage of the biomass is often, but not always, required (Murray, *et al.*, 1984, Sall, *et al.*, 1988, Boulton, 1991, O'Connor-Cox, 1997). Yeast is usually stored in designated yeast collection vessels (YCV's) as a slurry under spent wort at a temperature of 3-4 °C with gentle agitation, in order to ensure effective temperature control via external cooling jackets, to promote homogeneity, to prevent contamination and to minimize metabolic activity and cellular deterioration (Boulton, 1991, Rhymes & Smart, 2001, Gibson, *et al.*, 2007, Briggs, *et al.*, 2009, Somani, *et al.*, 2012). The permissive length of storage is influenced by the conditions applied and the physiological state of the yeast at the time of cropping. Particularly, the use of more concentrated worts in high gravity brewing (Section 1.3.6) may result in low viability yeast crops (Nakao, *et al.*, 2009, Dekoninck, *et al.*, 2012, Lei, *et al.*, 2012) and the strategy of yeast storage may be adjusted accordingly.

1.1.2.7 Acid washing

Low levels of bacterial contamination are often endured during yeast handling in the brewery, since completely sterile conditions are not always economically feasible. Providing the level of infection is low and the fermentation is vigorous, the influence of contaminants can be minimal (Boulton & Quain, 2001). However, yeast slurries can be washed with food grade

acids, such as phosphoric acid (pH 2.2), to remove bacteria (Cunningham & Stewart, 1998). As yeasts are more tolerant to acid condition than common bacteria, this functions to kill unwanted microorganisms prior to pitching without harming the yeast culture (Stewart, 1996, O'Connor-Cox, 1997, Elks, *et al.*, 1998, Cunningham & Stewart, 2000, Lodolo, *et al.*, 2008). However, non-bacterial forms of contamination, including wild yeasts and petite mutants are unaffected by this process (Stewart, 1996).

1.1.2.8 Yeast autolysis

The term 'autolysis' describes a self-destruction process in yeast. It represents self-degradation of the cellular constituents of a cell by its own enzymes following the death of the cell. During the production of beer, the occurrence of yeast autolysis has a negative effect on overall beer characteristics, including taste, mouth-feel, colour, and foam quality (Driscoll, *et al.*, 2002, Blasco, *et al.*, 2011). These detrimental effects usually are mediated indirectly through the destruction of large molecules such as proteins and polypeptides, with the concomitant release of peptides and amino acids. Moreover, free fatty acids released during autolysis destabilize beer foam, further imparting distinctive flavours to beer (Driscoll, *et al.*, 2002). Nevertheless, it should be noted that in some circumstances, the release of small molecules, such as amino acids and nucleotides, may impact positively on beer taste and mouth-feel. The issues of autolysis are more prevalent in large scale brewing operations, since the brewers usually reuse their yeast multiple times to reduce costs (Section 1.1.2.3), which can lead to cell death and eventually autolysis. On the other hand, autolysis can occur due to stressful environmental conditions, such as high alcohol, low pH and other factors, some of which occur during high gravity brewing fermentations (Section 1.3.6). Consequently, management of the risks associated with autolysis is critical to beer quality and process efficiency. It is of important to monitor yeast autolysis routinely in order to

optimize beer foam quality and yeast activity in breweries (Driscoll, *et al.*, 2002, Blasco, *et al.*, 2011), especially for high gravity beers.

1.1.3 Post-fermentation processing

Once the primary fermentation (Section 1.1.2.4) is complete, the ‘green beer’ undergoes a period of maturation with some flavour adjustment (particularly with regard to diacetyl reduction, SO₂ and DMS). Chill-haze proofing treatments are also conducted, aimed at ensuring the colloidal stability of beer such that no haze will develop over time (Lodolo, *et al.*, 2008). Fining agents such as isinglass are sometimes used to aid the clarification (Baxter, *et al.*, 2007, Walker, *et al.*, 2007). Maturation is terminated by filtration, resulting in the production of ‘bright beer’, which is then transferred from large capacity storage tanks into kegs, bottles or cans, depending on the type of beer and the designated point of sale. Alternatively, certain styles of beer such as British real ales do not require filtration, and secondary conditioning can occur in bottle or cask. This may be achieved using yeast carried through with the beer, or by the addition of a small quantity of viable yeast cells, in order to provide extra CO₂ production and minor flavour contributions (Boulton & Quain, 2001, Van Zandycke, *et al.*, 2011).

1.2 Yeast

Yeasts are single celled eukaryotic microorganisms classified within the Fungi kingdom (Kutty & Philp, 2008). There are around 1,500 species which have been described in detail, although it is believed that this only represents in the region of 1 % of yeast species which exist in nature (Kurtzman & Fell, 2006). Yeasts have been exploited for thousands of years, principally due to their capacity to produce ethanol and carbon dioxide, which are important in the production of certain foods and beverages, including bread, wine, distilled spirits, soy sauce and beer (Kutty & Philp, 2008).

1.2.1 Brewing yeast

Similar to other processes involving yeast, such as baking and distilling, yeast is critical to the production of beer, affecting the character and flavour of final product. Two types of brewing yeast are typically used, historically classified based on flocculation behaviour, and these can be divided into ale and lager yeast strains (Lodolo, *et al.*, 2008).

Ale strains belong to the species *Saccharomyces cerevisiae* (Pedersen, 1986, Smart, 2007) and have historically been described as top-fermenting yeast due to their hydrophobic nature, which causes flocs to adhere to the rising carbon dioxide bubbles leading to accumulation of yeast biomass at the top of the vessel (Hinchliffe, *et al.*, 1985, Amory & Rouxhet, 1988). However, the current use of CCV's (Section 1.1.2.4), which promotes 'bottom cropping' (collection of yeast biomass from the cone of the vessel), has somewhat blurred this description (Hammond, 1993).

Lager, or bottom-fermenting yeast are widely accepted as belonging to the species *Saccharomyces pastorianus* (Vaughn-Martini & Martini, 1998, Smart, 2007). They have been shown to be an interspecific hybrid organism, displaying different combinations of genomes from a *S. cerevisiae* strain and a non-*S. cerevisiae* strain, probably either an ancestral *S. bayanus* yeast (Rainieri, *et al.*, 2006, Dunn & Sherlock, 2008, Nakao, *et al.*, 2009), or a *S. bayanus*-like strain belonging to the species *S. eubayanus* (Libkind, *et al.*, 2011). It has been demonstrated that the DNA of lager yeasts exhibit approximately 85-98 % homology to those of *S. cerevisiae* based on a BLASTn analysis between their nucleotide sequences (Bond, *et al.*, 2004). In contrast, the mitochondrial DNA of lager yeast is believed to be solely inherited from the *S. bayanus*-like parent (Groth, *et al.*, 2000, Rainieri, *et al.*, 2006, Nakao, *et al.*, 2009). Irrespective of their origins, two sub-groups (Saaz and Froberg) of *S. pastorianus* have been identified based on yeast genome type, indicating at least two hybridization events

have occurred during the evolution of lager strains (Liti, *et al.*, 2005, Dunn & Sherlock, 2008, Gibson, *et al.*, 2013).

Lager yeasts are distinct from ale strains in terms of their physiological characteristics, partly due to their hybrid nature. Ale strains have a maximum growth temperature of between 37.5 °C and 39.8 °C, whereas lager yeasts have a maximum growth temperature of between 31.6 °C and 34.0 °C (Walsh & Martin, 1977). Furthermore, lager yeasts are typically employed to conduct fermentations at 8-15 °C whereas ale yeast are used at 18-25 °C (Walsh & Martin, 1977, Boulton & Quain, 2001). Strains also differ in their capacity to assimilate sugars; lager yeasts utilize melibiose based on secretion of α -galactosidase (melibiase), while ale yeasts lack this ability (Barnett, 1981). Lager yeast also appear able to assimilate maltotriose more rapidly than ale strains during wort sugar utilization (Stewart, *et al.*, 1995) and transport fructose via a proton symport mechanism (Desousa, *et al.*, 1995). Furthermore, under aerobic conditions, lager strains metabolise galactose and maltose simultaneously whereas ale strains preferentially ferment maltose (Crumplen, 1993).

1.2.2 Industrial yeast versus laboratory yeast

Industrial yeast strains are different from laboratory strains in several respects (Briggs, *et al.*, 2009, Nakao, *et al.*, 2009). Firstly, laboratory strains of *S. cerevisiae* are typically haploid or diploid, referring to single or double compliment of DNA respectively. In contrast, industrial yeast strains are often polyploid in nature, commonly comprising three or four sets of DNA (triploid or tetraploid). They may also be aneuploid (chromosome number is not a multiple of the haploid number since one chromosome set is incomplete) or allopolyploid (genomes from two different species are present) (Kielland-Brandt, *et al.*, 1995, Briggs, *et al.*, 2009), which make their genetic structure complex, and the copy number of specific genes difficult to ascertain. Additionally, whilst laboratory strains are capable of producing sexual spores, most industrial

yeasts reproduce almost exclusively asexually (by budding) and display low spore viabilities and poor sporulation competence (Hammond, 1993, Kielland-Brandt, *et al.*, 1995). Such poor sexual performance may be explained by their polyploid nature, resulting in the lack of proper meiosis in such strains. As a result of these characteristics, genetic and molecular tools developed for analysis of laboratory strains are not always applicable for industrial strains. However, it is known that genetic complexity can contribute greatly to strain robustness and stability which, in this context, leads to a superior ability to produce beer (Mortimer, 2000, Briggs, *et al.*, 2009).

1.3 Yeast growth requirements

Wort contains a variety of nutrients, including sources of carbohydrates and nitrogen, as well as lipids, minerals and vitamins. Each of these groups is required either to generate energy, or to support the growth of yeast. Consequently wort nutrients play a significant role in determining fermentation performance and beer quality (Casey, *et al.*, 1984, Gardner, *et al.*, 2005, Ernandes, *et al.*, 2006, Lodolo, *et al.*, 2008), as well as impacting on the development of flavour compounds (Section 1.4.5.3).

1.3.1 Nitrogen

Wort provides yeast with a complex mixture of nitrogen compounds, typically accounting for 4-5 % of the total dissolved solids (Boulton & Quain, 2001). The main forms of nitrogen in wort are amino acids, ammonium ions and small di- and tri-peptides, which are collectively known as free amino nitrogen (FAN). The majority of FAN is utilized for protein formation in yeast cells and is required for yeast growth, metabolism and fermentation (Casey, *et al.*, 1984, Pierce, 1987, Pugh, *et al.*, 2005, Stewart, 2009). Consequently, the level and composition of FAN has a significant impact on yeast physiology as well as flavour production brewing fermentations (Sankh, *et al.*, 2011, Lei, *et al.*, 2012). The uptake of

nitrogenous nutrients in brewing yeast is an ordered process and amino acids can be divided into four groups (Table 1.1) based on the order of their assimilation from wort (Pierce, 1987). The amino acids belonging to Classes A and B are required principally for protein synthesis under anabolic metabolism and are processed by permeases not subjected to nitrogen catabolite repression. Amino acids in Class C are only taken up when Class A amino acids have been used and nitrogen catabolite repression is relieved. Class D amino acids consist solely of proline. Reports have suggested that this amino acid is not assimilated at all, since the mitochondrial oxidase required for the oxidation of this amino acid is repressed during fermentation (Wang & Brandriss, 1987). However, a more recent study using an industrial-scale lager fermentation system revealed that assimilation of proline may occur under certain circumstances, believed to be influenced by fermentation conditions and yeast strain (Gibson, *et al.*, 2009).

Table 1.1 Classes of wort amino acids in order of assimilation during fermentation (Pierce, 1987). Amino acids are taken up in the order A, B, C, D.

<i>Class A</i>	<i>Class B</i>	<i>Class C</i>	<i>Class D</i>
Arginine	Histidine	Alanine	Proline
Asparagine	Isoleucine	Ammonia	
Aspartate	Leucine	Glycine	
Glutamate	Methionine	Phenylalanine	
Glutamine	Valine	Tyrosine	
Lysine		Tryptophan	
Serine			
Threonine			

1.3.2 Lipids

Previous studies have demonstrated that lipids including sterols and unsaturated fatty acids (UFAs) are essential for yeast division and largely determine plasma membrane bilayer structure in growing cells (Prasad & Rose, 1986, Rosenfeld, *et al.*, 2003, Redon, *et al.*, 2009). The most abundant UFAs in yeast are oleic acid (18:1) and palmitoleic acid (16:1), and the most abundant sterol is ergosterol (Rosenfeld, *et al.*, 2003). Since there is little lipid (mainly 16:0 and 18:2 fatty acid) in hopped pitching wort (Boulton & Quain, 2001), lipid compounds are required to be formed by yeast cells during the initial phase of fermentation, when oxygen is available. Oxygen is required for the conversion of squalene into ergosterol and for the formation of double bonds in UFAs (Rattray, *et al.*, 1975, Rosenfeld, *et al.*, 2003).

1.3.3 Minerals

Minerals, such as calcium, copper, iron, magnesium and zinc, are utilized by yeast for multiple functions centred around yeast growth and fermentation (Aleksander, *et al.*, 2009). For example, trace levels of zinc are required for the functionality of many enzymes including alcohol dehydrogenase, which plays an important role in the terminal step of fermentative metabolism (Lodolo, *et al.*, 2008). Typically adequate levels of minerals can be found in hopped wort derived from brewing liquor and other raw materials, however, the application of high and very high gravity brewing (Section 1.3.6) can result in nutrient deficiencies in these minerals, which may necessitate wort supplementation (Gibson, 2011).

1.3.4 Wort carbohydrates

Gravity, expressed in degree Plato (°P), is a brewing term used to refer to the amount of dissolved extract (sugar; carbohydrates) in wort (Boulton & Quain, 2001). Carbohydrates are the major nutrients in wort, comprising 90-92 % of the total dissolved solids (Boulton & Quain, 2001). A typical sugar spectrum for 11-12 °P wort is shown in Table 1.2, containing

fermentable carbohydrates (which account for 70-80 % total wort sugars) such as glucose, fructose, sucrose, maltose and maltotriose, as well as un-fermentable carbohydrates (20-30 %), such as dextrins. Carbon metabolism by brewing yeast is discussed in greater details in Section 1.4.

Table 1.2 Typical carbohydrate spectra of 11-12 °P wort (adapted from Stewart, 2009)

<i>Carbohydrate</i>	<i>Percent composition (%)</i>
Glucose	10-15
Fructose	1-2
Sucrose	1-2
Maltose	50-60
Maltotriose	15-20
Maltotetraose	< 1
Dextrins	20-30

1.3.5 Adjuncts

Adjuncts comprise any added source of fermentable sugar, and include both solid and liquid supplements (Boulton & Quain, 2001). The use of adjuncts is a cost-saving approach which is widely employed in high and very high gravity brewing (Section 1.3.6) to increase the amount of carbohydrate present at the start of fermentation. Solid adjuncts, such as maize, rice or sorghum (Boulton & Quain, 2001), require processing and are usually incorporated into either milling (Section 1.1.1.2) or mashing (Section 1.1.1.3). In contrast, liquid adjuncts

are usually added directly to the copper in the form of various syrups without specific treatment (Boulton & Quain, 2001).

Increasing wort gravity (Section 1.3.6) by adding adjuncts can markedly increase wort carbohydrate concentration, however this practice can also lead to nutritional imbalance since adjuncts are typically depleted in other nutrients. In particular, the addition of syrup adjuncts can dilute the nitrogen content of the media (Section 1.3.1), affecting yeast cell growth and fermentation efficiency, as well as beer quality and stability (O'Connor-Cox & Ingledew, 1989, Lei, *et al.*, 2012). Moreover, the type of sugar adjunct can have a considerable impact on yeast physiology (Pidcocke, *et al.*, 2009, Dekoninck, *et al.*, 2012). Pidcocke, *et al.* (2009) demonstrated that worts enhanced with maltose syrup yield a more balanced fermentation when compared to those conducted using a glucose based adjunct; the former yielded higher cell numbers, improved wort fermentability, and a more favourable flavour profile. Although the precise reasons for these effects remain unknown, Dekoninck, *et al.* (2012) produced evidence to suggest that yeast exposed to sucrose-supplemented high gravity wort experienced more osmotic stress (and other stress factors related to stationary phase) than comparable worts supplemented with maltose.

1.3.6 Yeast and high gravity brewing

1.3.6.1 Concept and advantages

In traditional brewing, worts of approximately 11-12 °P (corresponding to 11-12 grams extract per 100 grams liquid) are fermented to produce beers with 4-5 % (v/v) ethanol (Blieck, *et al.*, 2007). The term 'High Gravity (HG) brewing' is used to describe the practice of fermenting at approximately 15-20 °P, followed by dilution with water (usually carbon filtered and deoxygenated) to yield a finished beer with a regular alcohol content (i.e. 4-5 %, v/v) (Blieck, *et al.*, 2007, Stewart, 2010). Originally developed in the United States in the

early 1960s, HG brewing has been adopted as standard practice in many breweries (Boulton & Quain, 2001, Stewart, 2009). Due to the success of this approach, many brewers are exploring the possibility of 'Very High Gravity (VHG) brewing', a terminology currently used to describe fermentations conducted using wort within the range of around 20-25 °P (Gibson, 2011).

There are a number of advantages to high gravity brewing compared to using more 'traditional' worts. The use of higher gravity worts can increase the brewery capacity by improving the efficiency of plant facilities, and can also reduce capital expenditure, energy usage and labour costs (Whitaker & Crabb, 1977, Stewart, 2009, Puligundla, *et al.*, 2011). Secondly, the higher ethanol concentration produced from high gravity brewing promotes the precipitation of polyphenol and protein materials, resulting in reduced haze formation and increased microbiological stability in beer (Whitaker & Crabb, 1977). On a similar note, beers from high gravity brewing have also been reported to be smoother in taste (Hackstaff, 1978), possibly due to the loss of polyphenols. Moreover, high gravity brewing offers greater flexibility in product type, with the potential for dilution of a concentrated 'mother' beer for the creation of a range of products (Stewart, 2009).

1.3.6.2 Issues associated with high gravity brewing

Although an improved yield, in terms of ethanol production, can be obtained from HG and VHG brewing, fermentation cycle times are often increased and the productivity per unit time per vessel is correspondingly decreased, assuming all other fermentation parameters remain constant (Boulton & Quain, 2001). Therefore, one of the major management challenges in high gravity brewing centre on cost reduction of the protracted cycle times, without compensating final beer quality.

With regard to beer quality, a general concern is the change in flavour profile which can occur due to the modified wort composition. The most commonly used method to prepare concentrated wort is to supplement wort with sugar syrup adjuncts (Boulton & Quain, 2001, Reilly, *et al.*, 2004). As described previously (Section 1.3.5), this approach not only increases the carbohydrate content but also dilutes the available nutrients in wort, especially nitrogen, resulting in a change in carbon to nitrogen ratio, and a consequential shift in the concentration of flavour metabolites produced during fermentation (Lekkas, *et al.*, 2007, Sankh, *et al.*, 2011, Lei, *et al.*, 2012). Additionally, the dissolved oxygen (DO) is diminished further due to the decreased solubility of oxygen with increased wort gravity (Baker & Morton, 1977, Boulton & Quain, 2001). Insufficiently oxygenated wort can lead to poor yeast growth due to its role in lipid synthesis (Section 1.3.2) and inefficient sugar consumption (Casey, *et al.*, 1984). Moreover, a lack of foam stability has also been reported in beer from high gravity fermentations, most likely due to the proportionally lower concentration of hydrophobic polypeptides, compounds which act to form the backbone of foam (Cooper, *et al.*, 1998, Brey, *et al.*, 2002, Bamforth, 2006). Finally, the problem of residual sugars in beer becomes more serious when HG and VHG worts are applied, potentially resulting in economic losses related to decreased ethanol yield from wort carbohydrate and inappropriate organoleptic properties (Vidgren, *et al.*, 2009).

With regard to yeast fitness, the negative effects of high gravity brewing are associated with the stressful conditions which yeast cells encounter during the process. It has been reported that when compared to traditional gravity brewing, yeast used for HG or VHG fermentations are subjected to high levels of oxidative stress (Parrou, *et al.*, 1997), nutrient limitation (Casey, *et al.*, 1983, Lei, *et al.*, 2012), ethanol toxicity (Damore, *et al.*, 1990, Pratt, *et al.*, 2003), and osmotic stress (Hounsa, *et al.*, 1998, Pratt, *et al.*, 2003, Sigler, *et al.*, 2009). Yeast stress may also be induced by the greater amounts of carbon dioxide produced, since this

escapes more slowly from viscous high gravity wort (Thomas & Ingledew, 1995) and can be toxic to yeast cells at high concentrations (Lodolo, *et al.*, 2008). Each of these stress factors can have a profound influence on both yeast performance and the quality of the recovered biomass such as yeast autolysis (Section 1.1.2.8), and consequently the potential for yeast repitching (Section 1.1.2.3) can also be limited (Stewart, 2009). In order to reduce or eliminate these potential negative effects on yeast, it is necessary to understand yeast central carbon metabolism (Section 1.4) in conjunction with the mechanisms which cells can employ in order to adapt to extreme conditions (Section 1.6).

1.4 Yeast central carbon metabolism

Yeast cells undertake a series of complex reactions during brewery fermentations, resulting in cell division and growth of the population, and the concomitant conversion of wort nutrients into beer. In particular, the flow of carbon from the wort to the final products via central carbon metabolism is critical, since it determines the efficiency of the brewing fermentation and contributes to many of the major aspects of beer quality.

1.4.1 Assimilation of wort carbohydrates

Brewing yeast has the capacity to utilize a wide variety of wort sugars, the major exceptions being maltotetraose and dextrins (Stewart, 2009). Carbohydrates are taken up in a sequential manner (Boulton & Quain, 2001, Gibson, *et al.*, 2008), based largely on the ease at which they can be catabolised, transported and utilised internally, although the order of uptake is not absolute and some overlap may occur (Stewart, *et al.*, 1983). Sucrose is hydrolysed first by an extracellular invertase (β -d-fructosidase), located in the cell periplasm (Esmon, *et al.*, 1987, Stewart, 2009), resulting in an increase in its derivative sugars: glucose and fructose. Although its hydrolysis products are preferentially transported into the yeast cell, direct uptake of sucrose has also been observed in *S. cerevisiae* (Mwesigye & Barford, 1996,

Batista, *et al.*, 2004). Yeast cells then assimilate the simplest sugar monosaccharides first (glucose and fructose), followed by the more complex sugar disaccharide (maltose) and trisaccharide (maltotriose).

Glucose and fructose are the preferred carbon sources for brewing yeast and both are transported across the cellular membrane by multiple hexose transporters (Figure 1.2) (Ernandes, *et al.*, 1993, Reifenger, *et al.*, 1997, Ozcan & Johnston, 1999, Wiczorke, *et al.*, 1999). Glucose may inhibit fructose uptake due to a higher affinity of hexose transporters for glucose than for fructose (Verstrepen, *et al.*, 2004). Consequently most brewing strains consume glucose faster than fructose, although some strains utilize both monosaccharides at the same rate (Meneses, *et al.*, 2002). As noted earlier (Section 1.2.1), lager strains transport fructose actively by proton symport as well as via facilitated diffusion, whilst ale strains exclusively employ facilitated diffusion (Desousa, *et al.*, 1995).

Maltose and maltotriose are the major sugars in wort and consequently the capacity to utilize these sugars is vital to brewing yeast (Stewart, 2009). Before dissimilation, maltose and maltotriose are carried into the yeast cell by independent energy-dependent permeases (Needleman, 1991, Stewart, *et al.*, 1995, Stewart, 2009) (Figure 1.2). The driving force for this transport is via a proton symport mechanism, which depends on an electrochemical transmembrane proton gradient generated largely by plasma membrane ATPase, resulting in a stoichiometry of 1 ATP/proton hydrolysed to ADP (Serrano, 1977, Van Leeuwen, *et al.*, 1992). Once inside the cell, maltose and maltotriose are hydrolysed by the shared α -glucosidase system to yield two and three units of glucose, respectively (Stewart, 2009). Three genes involved in the uptake of maltose have been characterised, encoding the α -glucosidase (MALS), maltose permease (MALT) and an activator which co-ordinately regulates the expression of the α -glucosidase and permease genes (Hu, *et al.*, 1999).

Expression of these genes is regulated by maltose induction and repressed by high glucose concentrations (Stewart, 2006). Generally yeast will commence assimilation of maltose and maltotriose when approximately half of the wort glucose has been taken up, with a slower uptake rate for maltotriose (Zastrow, *et al.*, 2001)

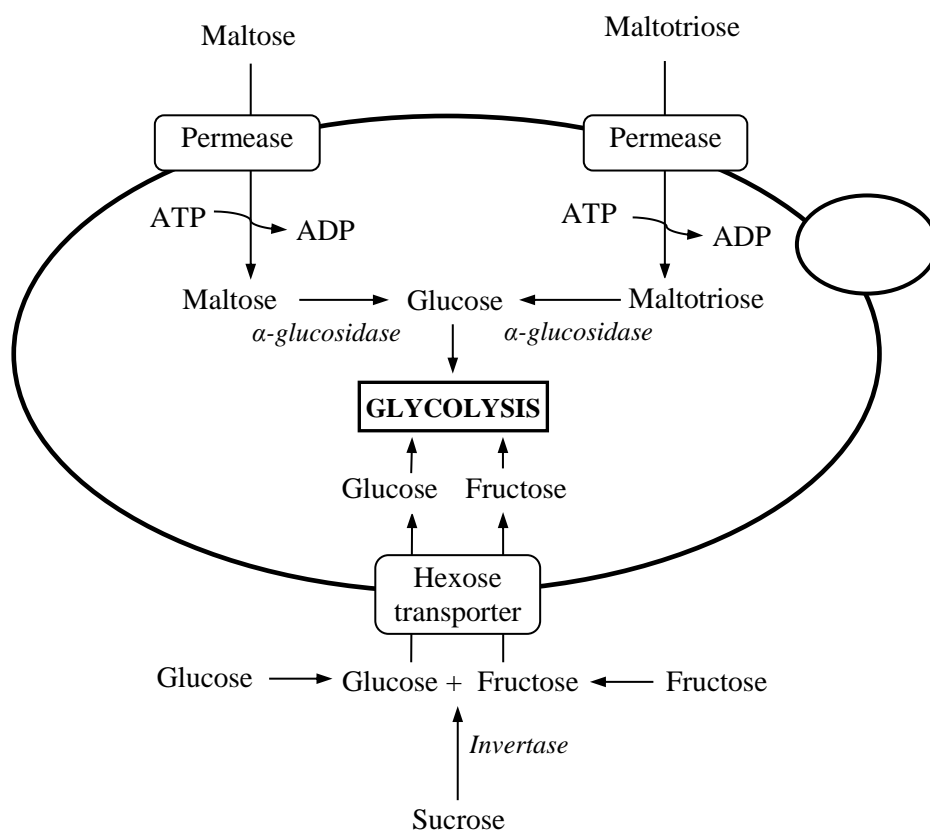


Figure 1.2 Mode of entry of wort sugars into the glycolysis pathway

1.4.2 Glycolysis

Glycolysis (Embden-Myerhof-Parnas pathway) is the principal route by which yeast cells break down glucose (Figure 1.3). During this process, one molecule of glucose is converted into two molecules of pyruvate, resulting in a net gain of two molecules of ATP as energy, and two molecules of NADH as reducing power (Briggs, *et al.*, 2009). A small portion of

glucose is catabolized through the pentose phosphate pathway (hexose monophosphate shunt) for the generation of NADPH which can be used for lipid synthesis (Barnett & Entian, 2005). However, in the case of brewing fermentations, this pathway is of relatively minor significance (Boulton & Quain, 2001).

In the EMP pathway (Figure 1.3), glucose-6-phosphate is initially produced by hexokinase activity on glucose with consumption of ATP, which is subsequently converted into its isomer fructose-6-phosphate by the action of phosphoglucose isomerase. Fructose-6-phosphate is then phosphorylated to yield fructose 1, 6-bisphosphate by the enzyme phosphofructokinase with consumption of ATP. Hydrolysis of fructose 1, 6-bisphosphate by the enzyme aldolase produces two three-carbon phosphates: glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. Glyceraldehyde 3-phosphate is oxidised to yield 1,3-disphosphateglycerate by glyceraldehyde 3-phosphate dehydrogenase, generating reducing power in the form of NADH. Subsequently, 3-phosphoglycerate and one molecule of ATP are obtained. The former is converted to 2-phosphoglycerate by the enzyme glycerophosphate mutase. Water is then removed by enolase, producing phosphoenolpyruvate. Finally, the product of glycolysis, pyruvate, is achieved by the activity of pyruvate kinase, with the release of another molecule of ATP (Briggs, *et al.*, 2009).

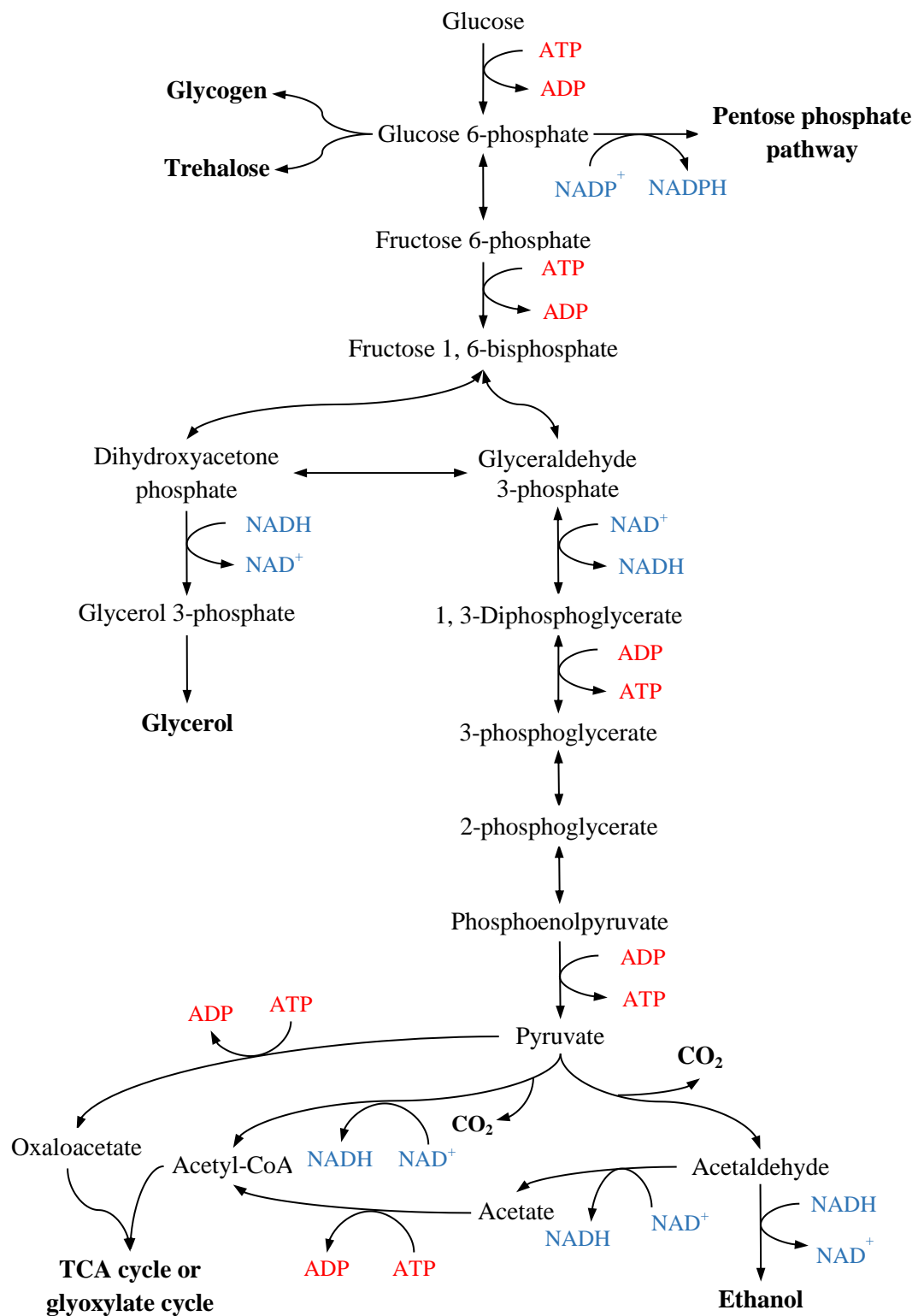


Figure 1.3 Schematic overview of glycolytic and fermentative pathway in yeast. Energy (ATP) production and consumption are shown in red, and reactions related to redox balance are shown in blue.

1.4.3 Role of pyruvate

The product of glycolysis, pyruvate, occupies a major junction between oxidative and fermentative sugar catabolism (Briggs, *et al.*, 2009). Furthermore, it may be involved in subsequent anabolic and catabolic pathways, leading to the formation of flavour-active metabolites (Section 1.5.5.3). There are at least three key reactions that direct the carbon flow beyond pyruvate (Figure 1.3): (I) the direct formation of acetyl-CoA, catalysed by pyruvate dehydrogenase and followed by oxidative reactions via TCA cycle; (II) the formation of acetaldehyde, catalysed by pyruvate decarboxylase and followed by the formation of ethanol; (III) the formation of oxaloacetate, catalysed by pyruvate carboxylase (Pronk, *et al.*, 1996) which can be used as an intermediate in TCA cycle and in the glyoxylate cycle as part of gluconeogenesis. In addition, a bypass route has been identified, where acetyl-CoA is derived indirectly from pyruvate via acetate, catalysed by acetaldehyde dehydrogenase and acetyl-CoA synthetase (Briggs, *et al.*, 2009).

In the brewing context, the direct conversion of pyruvate to acetyl-CoA and entry to the TCA cycle is repressed due to the presence of a high concentration of wort sugar (Section 1.4.4). Instead, the conversion of pyruvate to ethanol via acetaldehyde plays a dominant role; this pathway forms the major route for regeneration (re-oxidation) of NAD^+ (Figure 1.3), which is required in glycolysis (Section 1.4.2). Although the absolute requirement for acetyl-CoA production is reduced during fermentative metabolism, the bypass route through acetaldehyde and acetate ensures the synthesis of some acetyl-CoA, essential for biosynthetic reactions such as syntheses of fatty acids and amino acids (Briggs, *et al.*, 2009).

1.4.4 Carbon catabolite repression

During brewing fermentations, the presence of glucose and other wort sugars such as fructose and maltose has a significant effect on yeast carbon metabolism, including transcription,

repression of genes, and post-transcription repression of several protein systems. This occurs irrespective of the presence of oxygen (Briggs, *et al.*, 2009) and repressed cells are not capable of respiratory growth due to lack of a complete electron transfer chain. The TCA cycle is shut down because of the absence of the enzyme 2-oxoglutarate decarboxylase, and consequently fully active mitochondria are not observed in fermenting yeast (Piskur, *et al.*, 2006), potentially leading to the accumulation of respiration-deficient pro-mitochondria (Groot, *et al.*, 1972). This phenomena is termed carbon catabolite repression and is ultimately crucial to the outcome of the brewing fermentation (Boulton & Quain, 2001).

One effect of carbon catabolite repression is that sugars are taken up in a specific order as described previously (Section 1.4.1), but the major effect is to switch yeast metabolism from a respiratory to a fermentative mode. The latter is an inefficient method of energy transduction as only 2 molecules of ATP per glucose are produced, compared to a theoretical yield of 36 ATP molecule during respiration. As alluded to earlier, during the initial aerobic phase of fermentation, yeast cells exhibit a repressed physiology due to high sugar concentrations, known as the Crabtree effect, which enables the yeast to commence alcohol production (De Deken, 1966, Petrik, *et al.*, 1983). When the sugar content is reduced to non-repression levels, the anaerobic environment secures the fermentative mode and therefore the metabolism of yeast is continuously fermentative (Boulton & Quain, 2001, Briggs, *et al.*, 2009).

1.4.5 Direction of carbon flow

1.4.5.1 Production of ethanol

The flow of carbon from fermentable sugars to ethanol is the primary pathways exploited during brewing fermentation, with particular emphasis on this conversion in the case of high gravity brewing (Section 1.3.6). During typical batch fermentations there are progressive

changes in the rates of ethanol production, with an initial lag phase, corresponding with the passage of yeast from lag to exponential phase. During the latter stages of fermentation, the rate of ethanol formation reaches a maximum (Boulton & Quain, 2001). In traditional gravity brewing (11-12 °P), ethanol is produced within the range of 4-5 % (v/v) while in HG (15-20 °P) or VHG (20-25 °P) brewing (Section 1.3.6), ethanol concentration may reach higher than 10 % (v/v) (Briggs, *et al.*, 2009), which can affect yeast physiology and fermentation performance due to environmental stress factors (Section 1.5).

The plasma membrane is freely permeable to ethanol (Boulton & Quain, 2001) and consequently similar extracellular and intracellular concentrations of ethanol can be observed during fermentation (Panchal & Stewart, 1980, Damore, *et al.*, 1988). However, internal accumulation usually only occurs in early fermentation, where the rate of production is above the rate at which ethanol can diffuse out of the cells. As fermentation proceeds, the concentration of ethanol becomes equilibrated inside and outside the cell. Although an increase in intracellular ethanol concentration has been observed as the osmotic pressure of media increases (Panchal & Stewart, 1980, Damore, *et al.*, 1988), Damore, *et al.* (1988) concluded that nutrient limitation, rather than increased intracellular accumulation, was a major factor for the decreased growth and fermentation activities of yeast cells at higher osmotic pressures.

1.4.5.2 Release of carbon dioxide

During brewing fermentations, carbon dioxide is theoretically produced in equimolar proportions to ethanol (Boulton & Quain, 2001). However, the net yield of carbon dioxide is marginally lower since a small portion is utilized for anabolic carboxylation reactions in yeast cells (Oura, *et al.*, 1980). It has been demonstrated that dissolved carbon dioxide in fermenting medium is toxic to yeast cells (Lodolo, *et al.*, 2008), although sensitivity is strain-

dependent (Kruger, *et al.*, 1992). Super-saturation of carbon dioxide exacerbates the effect, resulting in the inhibition of cell growth, loss of cell viability and reduced fermentation rate (Kruger, *et al.*, 1992). Thus efficient nucleation and expulsion from the fermenting wort is required to minimize the impact on yeast (Kruger, *et al.*, 1992, Lodolo, *et al.*, 2008). Furthermore, if efficient pressure release systems are present within the fermenter, the liberation of carbon dioxide can be used to monitor fermentation progression (Boulton & Quain, 2001).

1.4.5.3 Formation of flavour compounds

In addition to the major fermentation products (ethanol and carbon dioxide), the flow of carbon to pyruvate and acetyl-CoA (Section 1.4.3) is also important due to the central role of these compounds in the formation of flavour-active metabolites (Boulton & Quain, 2001, Huang, *et al.*, 2010, Pires, *et al.*, 2014). It has been suggested that these pathways may be used due to cellular redox balancing reactions and a requirement for yeast cells to adapt to the changing environment during fermentation (Boulton & Quain, 2001). Interestingly, it has been demonstrated that yeast with desired flavour-producing characteristics may be identified based on the expression levels of flavour biosynthesis genes (Saerens, *et al.*, 2008).

Several hundreds of flavour compounds have been reported in beer (Pires, *et al.*, 2014), with varied thresholds (Meilgaard, 1975). Whilst these compounds are produced as the results of yeast metabolic mechanisms as described above, they can contribute to the overall flavour of the final beer, with both positive and negative perceptions. These include organic acids, fatty acids, higher alcohols and esters, as well as carbonyl and sulphur compounds. While higher alcohol and esters are generally accepted as desirable flavour components (depending on concentration and beer type), the carbonyl compounds known as vicinal diketones are typically regarded as off-flavours (Ashraf, *et al.*, 2010, Pires, *et al.*, 2014) as described in

more detail below.

Of the secondary metabolites, higher alcohols are generally produced in the greatest quantity by yeast cells. Apart from having importance to beer flavour and aroma, higher alcohols also play a vital role in providing precursors for ester synthesis (Boulton & Quain, 2001). The major compounds include n-propanol (solvent-like), isobutanol (alcoholic), 2-methyl-butanol (fruity, sweet), 3-methyl-1-butanol (fruity, sweet) and 2-phenylethanol (rose, floral). The carbon skeletons of the higher alcohols are supplied either via pyruvate (anabolic route), or from α -keto acids via the Erhlich pathway (Dickinson, *et al.*, 1997, Dickinson, *et al.*, 2003), which is directly linked to amino acid metabolism. Control of higher alcohol production has been reported to be influenced by manipulating *BAT1*-encoded branched-chain amino acid aminotransferases (Lilly, *et al.*, 2006) and *BAP2*-encoded branched-chain amino acid permease in brewing yeast (Kodama, *et al.*, 2001).

Esters are extremely important in determining the flavour profile of beer, and this group of compounds include ethyl acetate (fruity, solvent-like), isoamyl acetate (banana, apple), ethyl caproate (apple), and 2-phenyl ethyl acetate (rose, honey) (Verstrepen, *et al.*, 2003a). It is generally accepted that esters are formed from reactions between an alcohol (either ethanol or a higher alcohol) and a fatty acid acyl-CoA ester (Nordstrom, 1962, Suomalainen, 1981), catalysed by alcohol acetyltransferases. Three such enzymes, AATase I, AATase II and Lg-AATase II, have been found in yeast, encoded by *ATF1*, *ATFII*, and *Lg-ATF1*, respectively (Yoshimoto, *et al.*, 1998, Verstrepen, *et al.*, 2003b, Verstrepen, *et al.*, 2003c). The expression levels of *ATF1* and *ATF2* greatly affect the production of ethyl and isoamyl acetate, whereas *Lg-ATF1* is believed to have a more limited role (Verstrepen, *et al.*, 2003c). Additionally, two acetyl-CoA:ethanol O-acyltransferases, encoded by *EHT1* and *EEB1*, have been reported as

being responsible for the synthesis of medium chain fatty acid ethyl esters in yeast (Mason & Dufour, 2000, Saerens, *et al.*, 2006).

As mentioned previously, vicinal diketones (VDKs) belong to the carbonyl group and most are considered to have undesirable flavours. The two major VDKs are diacetyl (2,3-butanedione) and 2,3-pentanedione, which give a butterscotch-like flavour to beer (Meilgaard, 1975). Formation of these compounds is an indirect result of yeast metabolism via α -acetoxy acids involved in the biosynthesis pathway of valine and isoleucine. However, during later fermentation (the maturation stage), yeast reduces diacetyl to form acetoin and 2,3-butanediol, and reduces 2,3-pentanedione to form 2,3-pentanediol (Boulton & Quain, 2001). Such reduced forms have a higher flavour threshold and therefore less impact on beer flavour (Boulton & Quain, 2001). The relationship between these compounds and amino acid synthesis has been investigated through the valine content in the wort (Krogerus & Gibson, 2013), which can act as a means of decreasing VDK levels.

1.4.5.4 Biosynthesis of glycerol

Glycerol is a product of yeast metabolism which is created during fermentation in a redox-neutral process (Wang, *et al.*, 2001). It is synthesized in the cytosol of the yeast from the glycolytic intermediate dihydroxyacetone phosphate (DHAP) in two steps catalysed by glycerol-3-phosphate dehydrogenase (GPD) and glycerol-3-phosphatase (GPP) (Figure 1.3). Export of glycerol from cells is primarily controlled by an Fps1p channel protein under fermentative condition (Tamas, *et al.*, 1999) and the presence of glycerol in the final product can contribute to the body and mouth-feel of beer (Boulton & Quain, 2001), although it may be too low (usually 1-2 g/L) to contribute to the sensorial quality.

In addition to its sensorial quality, glycerol is generally considered to serve at least two important functions in yeast. (I) Formation of glycerol involves the conversion of NADH to

NAD⁺ (Figure 1.3), which functions to maintain the cytosolic redox balance especially under anaerobic conditions, acting as a sink for excess NADH produced by cellular reactions (Nordstrom, 1966, Schubert, 1998, Wang, *et al.*, 2001). This occurs when the yeast is actively growing and the flux from pyruvate through acetaldehyde to ethanol (Figure 1.3) is diminished such that the oxidation of NADH to NAD⁺ stalls (Boulton & Quain, 2008)(). This redox balancing role has been confirmed by the deletion of related genes, such as *GPD1* and *GPD2* (Nissen, *et al.*, 2000, Valadi, *et al.*, 2004, Hubmann, *et al.*, 2011). (II) Glycerol also plays an essential role as a compatible solute during osmoregulation in yeast (Blomberg & Adler, 1989, Wang, *et al.*, 2001, Taherzadeh, *et al.*, 2002, Hubmann, *et al.*, 2011). Typically yeast cells adapt to growth under increased osmotic pressure through the activation of High Osmolarity Glycerol (HOG) pathway (Section 1.6.3.2), which ensures the accumulation of glycerol to reduce the transmembrane difference of osmotic strength without any detrimental effects on cellular functions (Hohmann, 2002).

1.4.5.5 Accumulation of glycogen and trehalose

Glycogen and trehalose are two important carbohydrates found within yeast cells during fermentation. Synthesis of glycogen is derived from glucose, via a pathway involving glucose 6-phosphaste and glucose 1-phosphate, while trehalose may be synthesized from glucose or from glucosyl residues derived from degradation of glycogen (Figure 1.4).

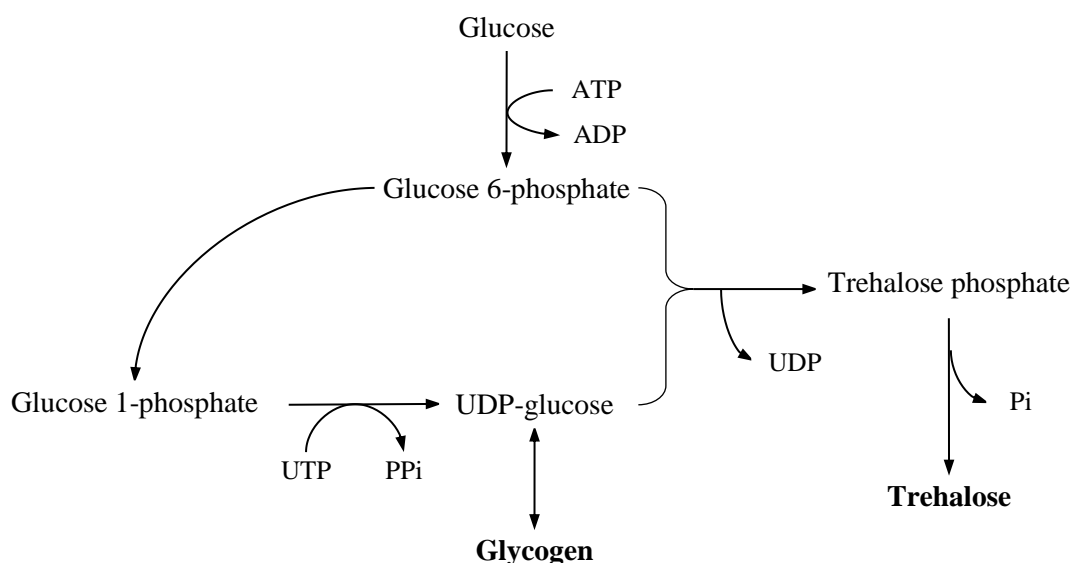


Figure 1.4 Biosynthesis pathways of glycogen and trehalose. UTP: Uridine triphosphate, UDP: Uridine diphosphate; PPi: inorganic pyrophosphate; Pi: inorganic phosphate

Glycogen is a multi-branched polysaccharide of D-glucose (Torija, *et al.*, 2005). In the context of the brewing fermentation, accumulation or degradation of glycogen is controlled by yeast growth rate and influenced by nutrient limitation (Boulton & Quain, 2001, Francois & Parrou, 2001). Glycogen not only serves as a source of carbon and energy for synthesis of sterols and unsaturated fatty acids during the aerobic phase of fermentation (Boulton & Quain, 2001), but also provides energy for cellular maintenance during both the stationary phase of fermentation and during storage of cropped yeast (Quain & Tubb, 1982, Somani, *et al.*, 2012). There are several genes involved in glycogen synthesis, including *GSY1* and *GSY2* (encoding two glycogen synthases) which play important roles in the formation of glycogen from individual glucose units (Gibson, *et al.*, 2008, Wilson, *et al.*, 2010, Dekoninck, *et al.*, 2012).

Trehalose is a non-reducing disaccharide consisting of two molecules of D-glucose. Accumulation of trehalose usually takes place after glycogen formation, since the repressive

effects of glucose (Section 1.4.4) are higher in the trehalose synthesis pathway than in glycogen synthesis pathway (Stewart & Russell, 1993). Genes involved in trehalose synthesis and degradation such as *TPS1* (encoding trehalose-6-phosphate synthase) and *TPS2* (encoding trehalose-6-phosphate phosphatase), and *NTH1* (encoding neutral trehalase), (Parrou, *et al.*, 1997, Parrou, *et al.*, 1999, Gibson, *et al.*, 2007) are regulated via the stress response element (STRE) (Section 1.6.3.3). Although trehalose acts as a storage polysaccharide (Lillie & Pringle, 1980), its primary function is not energy related, but as a stress protectant to guard cells against a range of adverse environmental conditions, including heat shock (Hottiger, *et al.*, 1987, Odumeru, *et al.*, 1993, Mahmud, *et al.*, 2010), osmotic stress (Sharma, 1997, Hounsa, *et al.*, 1998), and ethanol toxicity (Odumeru, *et al.*, 1993, Mahmud, *et al.*, 2010). Although the protective effect of trehalose has not been well explored at the molecular level (Mahmud, *et al.*, 2010), trehalose has been shown to be an important membrane stabilizer (Crowe, *et al.*, 1984) and, in order to function with maximum effect, is required to be present at both the inner and outer surface of the membrane (Crowe, *et al.*, 1984). This is achieved by a cellular trehalose/H⁺ symport mechanism (Kotyk & Michaljanicova, 1979), which transports trehalose from the site of synthesis in the cytosol to the membrane (Eleutherio, *et al.*, 1993).

1.4.5.6 Yeast biomass

During brewing fermentations, yeast cells typically divide between 2-4 times, depending on the pitching rate and the fermentation conditions (Ferreira, *et al.*, 2010). After fermentation the remaining particulate matter includes beer solids, trub solids and yeast biomass (Ferreira, *et al.*, 2010). In order to achieve maximum fermentation efficiency and the desired beer quality, fermentation management is generally required to maintain a balanced nutrient distribution between the yield of ethanol and yeast biomass, as well as to a lesser extent other metabolites. Therefore yeast proliferation and growth rate are controlled through

fermentation conditions, where the levels of oxygen supplied at the beginning of fermentation is usually a key determinant (Boulton & Quain, 1985, Boulton & Quain, 2001).

1.4.5.7 Fermentation mass balance

Previous researchers have attempted to use fermentation products to calculate a fermentation mass balance. Antoine Lavoisier first described this phenomenon in 1790 when he realized that sugars are transformed into carbonic acid, alcohol and yeast biomass after fermentation (Lavoisier, 1790). Subsequently, Karl Napoleon Balling (1845) published a fermentation mass balance formula based on the concept that fermentable wort solids contribute to yeast mass increase. This formula has been accepted as a standard ASBC method (Beer-6A, 2014) and has been applied in brewing practice for over 100 years (De Clerck, 1958, Nielsen, 2004), allowing a level of control over beer production. However, there are two assumptions used in the derivation of Balling's formula: (I) 0.11 g of carbohydrate is converted to yeast mass for each gram of ethanol produced in fermentation. (II) All fermentable dissolved wort solids are monosaccharides. More recently, Cutaia (2007) described an improved measurement of yeast proliferation during brewing production, taking into consideration of four wort carbohydrates (glucose, fructose, maltose and maltotriose) and limitation factors of yeast growth due to sterols and dissolved oxygen. This author concluded that Balling's classic formula can yield inaccurate estimates especially when the wort composition significantly departs from traditional practices, such as the use of adjuncts and novel aeration system in high gravity brewing (Section 1.3.6).

1.5 Stresses associated with brewing fermentation

During beer production, yeast cells are exposed to multiple stress factors (Figure 1.5) including temperature shocks (Hottiger, *et al.*, 1987, Somani, *et al.*, 2012), high oxidative values (Parrou, *et al.*, 1997, Belinha, *et al.*, 2007), nutrient limitations (Casey, *et al.*, 1983,

Lei, *et al.*, 2012), ethanol toxicity (Damore, *et al.*, 1990) and osmotic stress (Hounsa, *et al.*, 1998, Pratt, *et al.*, 2003, Sigler, *et al.*, 2009). Furthermore, with wort gravity increases (Section 1.3.6), many of these stress factors become correspondingly pronounced (Stewart, 2009, Puligundla, *et al.*, 2011).

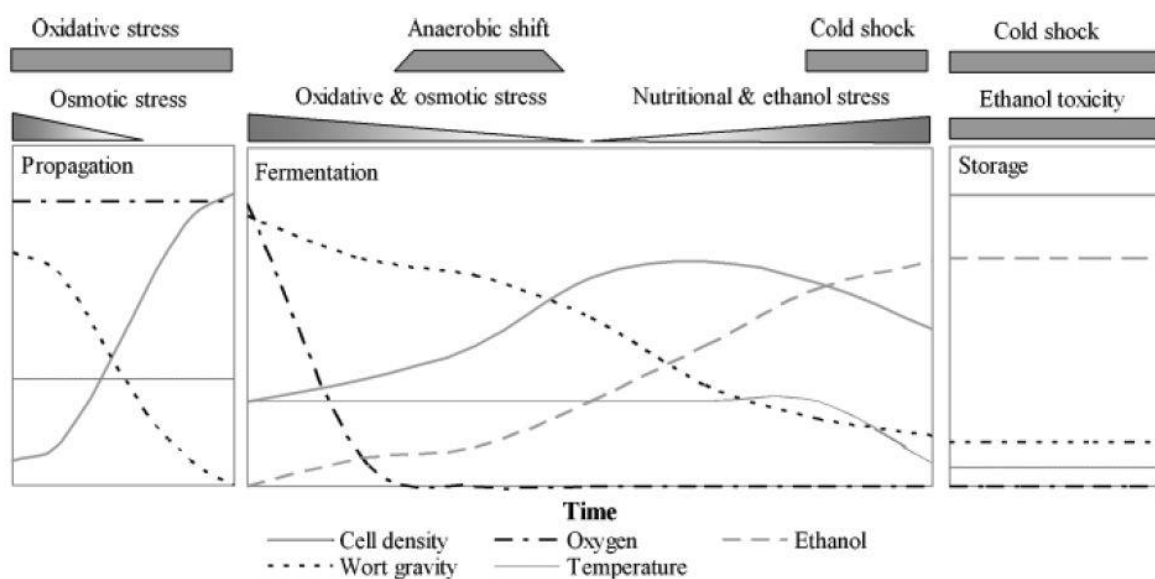


Figure 1.5 The presence of multiple stress factors encountered by yeast cells during yeast propagation, fermentation and storage (Gibson, *et al.*, 2007)

Temperature stress is believed to be induced during the switch from fermentation to storage environment (Kondo & Inouye, 1991, Kondo, *et al.*, 1992, Kowalski, *et al.*, 1995). Although lager fermentations are conducted at relatively low temperatures (8-15 °C), ale strains are often pitched into worts of 20 °C. Irrespective, once fermentation is complete, cold shock may be induced as yeast cropping and storage is typically conducted at 3-4 °C (Briggs, *et al.*, 2009).

Yeast cells may also be subjected to nutrient limitation towards the end of fermentations. In particular, the use of HG and VHG worts (Section 1.3.6) may also cause additional nutrient

limitation characterised by limited FAN and lipid deficiency, due to the addition of syrup and insufficient dissolved oxygen, respectively (Casey, *et al.*, 1983, Lei, *et al.*, 2012) (Section 1.3.6.2). Such fermentations are often associated with inefficient cell growth, abnormal patterns of sugar uptake and undesirable flavour profiles (Lei, *et al.*, 2012).

At the end of fermentation, high ethanol concentration, especially in HG and VHG brewing (Section 1.3.6), can also cause stress (Damore, *et al.*, 1990, Pratt, *et al.*, 2003). Ethanol toxicity has been described as targeting both non-specific (Jones & Greenfield, 1987) and specific sites, including the cell membrane (Jimenez & Benitez, 1987, Salgueiro, *et al.*, 1988, Alexandre, *et al.*, 1994, You, *et al.*, 2003) and the mitochondria (Bandas & Zakharov, 1980, Aguilera & Benitez, 1985, Aguilera & Benitez, 1989, Kitagaki, *et al.*, 2007).

Previous reports have also suggested that oxidative stress may be induced during aerobic propagation and at the beginning of fermentation, when cells are transferred (either from propagator or from anaerobic storage) into oxygenated or aerated wort. In the case of HG and VHG brewing (Section 1.3.6), wort oxygenation is particularly important and increased oxygenation rates in such scenarios have also been implicated in elevated oxidative stress (Boulton & Quain, 2001). This is due to the production of reactive oxygen species (ROS) such as hydrogen peroxide and the hydroxyl radicals (OH[•]) (Halliwell & Gutteridge, 1999). These act to oxidize nucleic acids, proteins, lipids and carbohydrates, resulting in damaged membrane activity and cellular functions (Belinha, *et al.*, 2007). Finally, osmotic stress is one of the major environmental stresses associated with HG and VHG brewing (Section 1.3.6) and will be discussed in more details in the following section (Section 1.6).

1.6 Osmotic stress and the osmotic response of brewing yeast

1.6.1 Sources of osmotic stress

Osmotic stress is associated with any situation where there is an imbalance of intracellular and extracellular osmotic potential sufficient to cause a deleterious change in physiology (Csonka & Hanson, 1991). Hypo-osmotic stress refers to a low external osmotic potential, resulting in an influx of water into cells (Dihazi, *et al.*, 2001), whereas hyper-osmotic describes the high environmental solute concentration, characterized by the loss of cellular water and subsequent turgor (Wood, 1999, Wojda, *et al.*, 2003). The latter is more relevant to the brewing environment and is the focus of the current study.

It has been suggested that two principal sources of hyper-osmotic stress exist in the brewing process (Gibson, *et al.*, 2007). The first occurs during the practice of acid washing (Section 1.1.2.7), where yeast slurries are submerged in food grade acids to achieve pH ranging 2.2-2.5 and osmotic stress is induced by the abundance of dissociated H⁺ ions (Gibson, *et al.*, 2007). The second source involves the process of yeast pitching (Section 1.1.2.3), where cells are inoculated into wort and osmotic stress is induced due to the high concentration of sugars present (Briggs, *et al.*, 2009). The use of HG or VHG wort, with elevated sugar concentrations, has been suggested to exacerbate the external osmotic stress to the detriment of yeast cells, such as the loss of yeast viability (Damore, 1992, Cahill, *et al.*, 2000, Dekoninck, *et al.*, 2012), the reduction of mean cell volume (Pratt, *et al.*, 2003), enlargement of yeast vacuole and changes in the topography of the yeast cell surface (Pratt, *et al.*, 2007, Stewart, 2010).

1.6.2 Measurement of external osmotic pressure

Osmotic concentrations can be expressed as either osmolality or osmolarity (Erstad, 2003, Barr & Pesillo-Crosby, 2008). The latter is a measure of the number of moles of osmotically active particles per liter of solution (Osm/L) (Equation 1.1), a value which is difficult to determine as the volume of solution changes with the amount of solute added, as well as with changes in temperature and pressure. Similar in concept, osmolality determines the number of moles of osmotically active particles per kilogram of solution (Osm/kg) (Equation 1.2). This measure is easier to evaluate and more commonly used in practical osmometry, since the amount of solvent will remain constant regardless of changes in temperature and pressure (Erstad, 2003). In typical biological solutions, the difference between osmolality and osmolarity is negligible and most sources use the terms interchangeably (Erstad, 2003, Barr & Pesillo-Crosby, 2008). The relationship between osmolality and osmolarity is demonstrated in Equation 1.3. In a fixed system where the volume of solution and solvent remains constant, the ratio of osmolality and osmolarity is a positive constant and hence their relationship is linear. It should be noted that values for osmolality must be higher than for osmolarity, since the ratio is greater than 1. It should also be stated that the osmotic pull of a solution (e.g. osmolality) is influenced by the total number of the dissolved solute particles but not by their size or shape in the solution (Sweeney & Beuchat, 1993, Barr & Pesillo-Crosby, 2008).

An osmometer is a device used to measure the osmotic strength of a solution in the form of osmolality and expressed in mOsm/kg. There are three types of osmometer commercially available, including freezing point osmometer (FPO), vapor pressure osmometer (VPO) and membrane osmometer (MO). FPO and VPO both provide rapid and inexpensive measurements requiring a small sample size. Whilst VPO cannot be used for volatile

compound such as alcohols, FPO is ideally suitable for most biological and aqueous applications based on industry preferred freezing point method (Koumantakis & Wyndham, 1989). Furthermore, although MO provides direct measurement of osmolality, it requires a long analysis time and a large sample volume.

Equation 1.1 Definition of osmolarity as a measurement of osmotic concentration

$$\text{Osmolarity} = \frac{\text{osmoles of solute (Osm)}}{\text{Volume of solution (L)}}$$

Equation 1.2 Definition of osmolality as a measurement of osmotic concentrations

$$\text{Osmolality} = \frac{\text{osmoles of solute (Osm)}}{\text{weight of solvent (kg)}}$$

Equation 1.3 Relationship between osmolality and osmolarity

$$\begin{aligned} \frac{\text{Osmolality}}{\text{Osmolarity}} &= \frac{\text{volume of solution (L)}}{\text{weight of solvent (kg)}} = \frac{\text{volume of solution (L)}}{\text{volume of solvent (L)} \times \text{density of solvent (kg/L)}} \\ &= \frac{\text{volume of solution}}{\text{volume of solvent}} = C > 1 \end{aligned}$$

Where solvent (water) density equals to 1 kg/L and C means constant

1.6.3 Osmophilic yeast

Osmophilic or osmotolerant yeast has been designated to yeast that are able to grow in highly concentrated organic solutes, particularly sugars (Munitis, *et al.*, 1976). These organisms do not require a high water activity value and tolerate drier environments than non-osmotolerant

organisms. Many of the yeasts belong to the genus *Zygosaccharomyces*, particularly *Zygosaccharomyces rouxii* (Martorell, *et al.*, 2007, Leandro, *et al.*, 2011, Watanabe, *et al.*, 2013), as well as *Zygosaccharomyces bailii* (Thomas & Davenport, 1985, Steels, *et al.*, 1999, Martorell, *et al.*, 2007) and *Zygosaccharomyces lentus* (Steels, *et al.*, 1999). Martorell, *et al.* (2007) reported that *Z. rouxii* strains are able to grow at 90 % (w/v) glucose, vigorously ferment hexose sugars, grow at low pH, tolerate high temperatures and grow at high molar NaCl concentrations. Vanzyl, *et al.* (1990) once suggested that *Z. rouxii* cells protect themselves during osmo-regulation by the synthesis of osmo-protectants such as glycerol and more recently, Watanabe, *et al.* (2013) demonstrated that the organisms adapt themselves to this high osmotic environment through copy number amplification of *FLO11D* (a gene responsible for floc formation and that its expression is induced by osmotic stress). The authors found that the *Z. rouxii* strain with a higher copy number of *FLO11D* displays a fitness advantage compared to a reference strain under osmotic stress static conditions. Moreover, *Z. bailii* and *Z. lentus* were found to be preservative-resistant and capable of growth at low temperature, low pH, high sugar foods and drinks (Steels, *et al.*, 1999). Because of this, the osmophilic yeasts can usually cause spoilage of honey, jams, molasses, sugar syrups, soft drinks, concentrated fruit juices and wines (Thomas & Davenport, 1985). Although no osmophilic organisms are highly pathogenic, they may cause food poisoning and opportunistic infections, especially in people with weakened immune systems. Consequently, whilst it is of great importance in the food industry to avoid such osmophilic yeast, it is proposed that the yeast may be considered as a benchmark to the behaviour of *Saccharomyces* yeasts in high gravity fermentations in future study.

1.6.4 Yeast response to osmotic stress

1.6.4.1 General overview

In order to respond to hyperosmotic stress, yeast is known to display a series of active processes by which cells monitor and adjust osmotic pressure in order to regain turgor, repair cellular damage and resume growth (Morris, *et al.*, 1986, Mager & Siderius, 2002, Wojda, *et al.*, 2003): (I) As an immediate consequence of a sudden exposure to an osmotic environment, cells rapidly lose intracellular water, leading to the decrease of cell volume and turgor and hence the shrinkage of cells (Morris, *et al.*, 1986, Meikle, *et al.*, 1988, Marechal & Gervais, 1994, Pratt, *et al.*, 2003). This process is driven by physico-mechanical forces, and the ability to withstand osmotic pressure may largely depend on the intrinsic cellular attributes of individual yeast, for example ‘superior’ membrane structure (Sharma, *et al.*, 1996) and vacuolar functioning (Morris, *et al.*, 1986, Latterich & Watson, 1993, Nass & Rao, 1999, Pratt, *et al.*, 2007). (II) The primary osmotic response is triggered by the cellular changes described above; firstly cell growth arrest is observed at both G1 (Belli, *et al.*, 2001) and the G2/M transition stages of the cell cycle (Alexander, *et al.*, 2001). Subsequently, the glycerol channel Fps1p (Tamas, *et al.*, 1999) closes followed by the activation of the high osmolarity glycerol (HOG) pathway (Hohmann, 2002, Saito & Tatebayashi, 2004) (Section 1.6.3.2), leading to the accumulation of the major compatible solute glycerol (Section 1.4.5.4). Part of these molecular events overlaps with the general stress response (Section 1.6.3.3), where transcription factors Msn2/Msn4p act as mediators (Martinez-Pastor, *et al.*, 1996, Watanabe, *et al.*, 2011). (III) The yeast cells which survive after the primary phase of response reach a ‘sustained’ phase of response (Mager & Siderius, 2002). The cellular accumulation of glycerol results in the intake of water, swelling of the cell with a resulting increase in size, and the recovery of turgor (Morris, *et al.*, 1986, Hohmann, 2002, Mager & Siderius, 2002).

When a critical cell size has been achieved, cellular damage can be repaired and normal cell functions can resume (Mager & Siderius, 2002).

1.6.4.2 High osmolarity glycerol (HOG) pathway

In yeast cells, the high osmolarity glycerol (HOG) pathway (De Nadal, *et al.*, 2002, Hohmann, 2002, Zi, *et al.*, 2010) is central to the osmotic response. This signalling pathway has been shown to be induced by two plasma membrane proteins (Sho1 and Sln1) (Figure 1.6), which are responsible for sensing environmental osmotic changes (Hohmann, 2002). Signals from each protein are transduced by independent components and converge to initiate the conserved section of the HOG pathway (O'Rourke & Herskowitz, 2004). The Sho1 branch requires Cdc42, Ste20 and Ste50 to activate Ste11 (Maeda, *et al.*, 1995, Raitt, *et al.*, 2000, Reiser, *et al.*, 2000) whereas the Sln1 branch uses Ypd1 and Ssk1 to activate Ssk2 and Ssk22 (Posas & Saito, 1998, Reiser, *et al.*, 2003). Any of the three resultant mitogen-activated protein kinase kinase kinases (MAPKKK) (Ste11, Ssk2 or Ssk22) are able to activate the mitogen-activated protein kinase kinase (MAPKK) (Pbs2), which is then phosphorylated to the mitogen-activated protein kinase (MAPK) (Hog1), leading to the translocation of Hog1 to the nucleus and an increase in its kinase activity (Brewster, *et al.*, 1993, Bilsland-Marchesan, *et al.*, 2000, Proft, *et al.*, 2001, Hohmann, 2002, Klipp, *et al.*, 2005, Zi, *et al.*, 2010) as described in Figure 1.6.

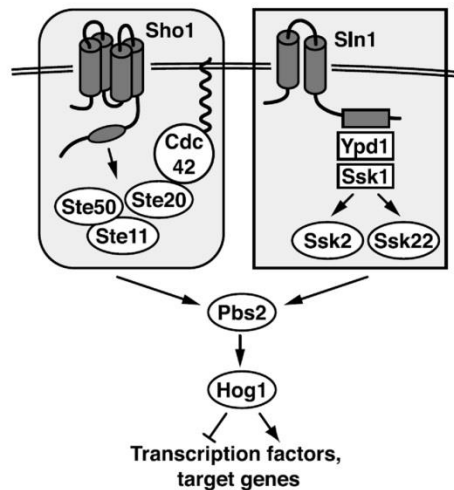


Figure 1.6 Activation of the high osmolarity glycerol (HOG) pathway induced by two plasma membrane proteins Sho1 and Sln1 (O'Rourke & Herskowitz, 2004)

Klipp, *et al.* (2005) described a comprehensive cellular signalling model upon hyperosmotic shock, revealing the precise mechanism of the HOG pathway (Figure 1.7), and dividing it into four inter-related modules. Under increased external osmotic stress, the signalling system is initiated by the inactivation of Sln1 (Reiser, *et al.*, 2003), leading to the accumulation of Ypd1 and Ssk1, and then the activation of Ssk2 and Ssk22. Subsequently the MAPKK Pbs2 and MAPK Hog1 are activated and the active Hog1 stimulates expression of specific genes in the nucleus. These genes include those involved in glycerol biosynthesis, such as *GPD1* and *GPP2*, and consequentially glycerol is produced through the conversion of dihydroxyacetonephosphate (DHAP) via glycerol-3-phosphate (Klipp, *et al.*, 2005). In addition, the HOG pathway also appears to mediate the activity of a sensitive glycerol channel, Fps1 (Luyten *et al.*, 1994; Tamás *et al.*, 1999), which reduces the efflux of glycerol and thus aids the accumulation of internal glycerol. Furthermore, the HOG pathway is required for the activation of Msn2p and Msn4p, indicating that the signalling system may have a role in regulating GSR via STRE (Section 1.6.3.3) (Schuller, *et al.*, 1994).

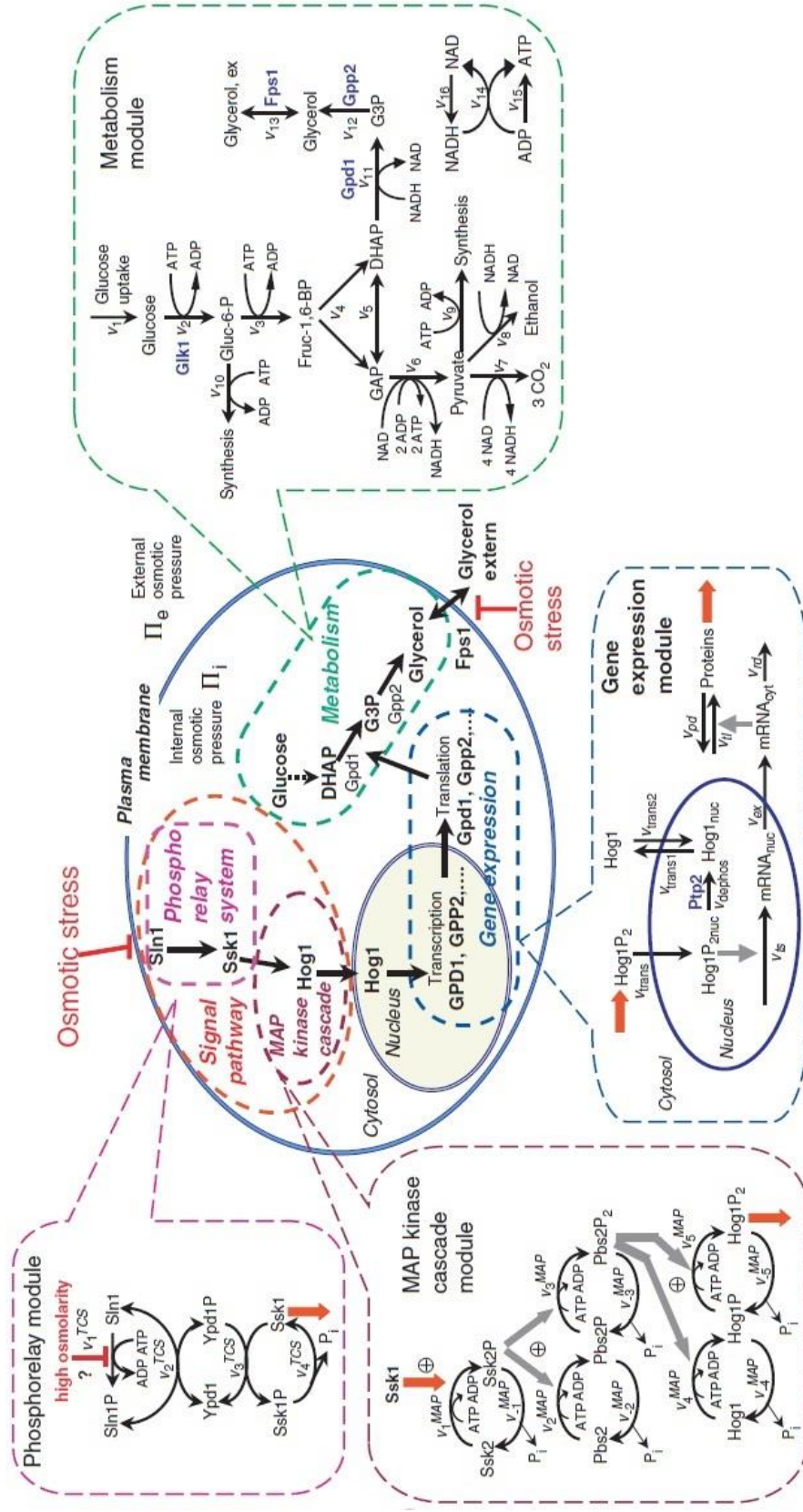


Figure 1.7 Mechanism of the High Osmolarity Glycerol (HOG) pathway upon hyper osmotic stress, including details of the phosphorelay module, MAP kinase cascade module, gene expression module and metabolism module (Klipp, *et al.*, 2005).

1.6.4.3 The general stress response (GSR)

There are two major stress response pathways in yeast cells which function to protect cells against a range of environmental challenges, including osmotic stress. One is the heat shock response (HSR) pathway, which is induced when cells are exposed to sub lethal heat shock as well as other stress factors such as heavy metals and anoxia (Mager & Dekruijff, 1995, Chatterjee, *et al.*, 2000). The other is the general stress response (GSR) pathway, which usually occurs due to a wider variety of adverse environmental conditions such as osmotic stress, oxidative stress, nitrogen starvation and low external pH (Ruis & Schuller, 1995, Chatterjee, *et al.*, 2000).

Regulation of the GSR system involves of induction of a wide range of genes (~200) functioning in a diverse assay of cellular behaviours (Ruis & Schuller, 1995, Gasch, *et al.*, 2000, Causton, *et al.*, 2001). These genes have been demonstrated to contain a general stress responsive element (STRE) (CCCCT or AGGGG) within their upstream regulatory region (Schmitt & McEntee, 1996), and two zinc finger transcriptional activators (Msn2p and Msn4p) are responsible for activation of STRE within the corresponding genes (Martinez-Pastor, *et al.*, 1996, Watanabe, *et al.*, 2011). STRE or STRE-like sequences have been identified in the promoter region of many stress-responsive genes such as *CTT1* encoding for yeast catalase T (Marchler, *et al.*, 1993, Verbelen, *et al.*, 2009b) and *HSP12* encoding for yeast heat-shock protein (Varela, *et al.*, 1995). It should be noted that Msn2p is degraded rapidly following the stress response and that GSR behaviour is regarded as a transient phenomenon (Bose, *et al.*, 2005).

1.7 Aims and objectives

The brewing industry, although often considered a traditional process, is dynamic and open to new developments in technology. To this end, HG and VHG brewing have largely been

implemented worldwide with financial gains. However, a major concern regarding this practice is the requirement to produce beer of maximum ethanol concentration without compromising the efficiency of beer production and the quality of final product. Yeast activity, and the conversion of sugar to ethanol and other metabolites via central carbon metabolism, is particularly important since it is the principle mechanism by which beer is produced. Simultaneously, elevated osmotic stress induced by HG and VHG wort poses a significant threat to yeast physiology and consequently fermentation performance. Therefore, the interrelationship between increased wort gravity, carbon flux, and the physiological effects of osmotic pressure during HG and VHG brewing needs to be better understood.

This thesis will firstly characterize two lager-type and two ale-type brewing yeasts based on their phenotypic and genomic differences. Subsequently, the central carbon flux of these strains will be explored using a series of HG and VHG fermentations, where the utilization of wort carbohydrates and the profile of key carbon metabolites will be determined, leading to an estimation of carbon partitioning from wort sugars to carbon products. Additionally, wort osmolality, as a measure of extracellular osmotic pressure, will be assessed during the course of fermentations, and the contribution of potential wort compounds to osmolality will be determined. Finally, the major effects of increased osmolality on yeast physiology, such as cell survival rate, membrane fluidity and DNA integrity, will be investigated to determine the challenges faced by yeast cells under HG and VHG conditions. The primary goal of this thesis is to provide a greater understanding of the yeast response to conditions associated with high gravity fermentations, potentially leading to process optimisation in the future.

CHAPTER 2: MATERIALS AND METHODS

2.1 Yeast strains

Two lager type yeast strains (Lager1 and W34/70) and two ale type strains (NCYC1332 and M2) were used in this study (Table 2.1).

Table 2.1 Yeast strains used in the study

<i>Strain</i>	<i>Source</i>	<i>Additional information</i>
Lager1	Molson Coors Brewing Company, UK	Proprietary lager strain
W34/70	Hefebank, Weiherstephan, Germany	Widely used lager strain
NCYC1332	National Collection of Yeast Cultures (NCYC), Norwich, UK	Ale strain
M2	University of Nottingham Yeast Culture Collection	A flocculent isolate of ale strain NCYC1168 (Isolated by M. Rhymes, Oxford Brookes University, UK)

2.2 Growth media and storage

All chemicals were obtained from Fisher Scientific (UK) unless stated.

2.2.1 YPD media

Each strain was maintained and grown on YPD media (2 % [w/v] bacteriological peptone, 1 % [w/v] yeast extract and 2 % [w/v] D-glucose in RO [reverse osmosis treated] water). If required, solid YPD media was prepared by the addition of 1.2 % (w/v) agar. Following preparation, media was sterilised by autoclaving at 121 °C and 15 psi for 15 min.

2.2.2 Cryogenic storage of yeast

Long term stock cultures of each strain were maintained at -80 °C. A loop-full of yeast, pre-grown on YPD agar, was suspended in 1 mL YPD media containing 25 % (v/v) glycerol as a cryoprotectant and transferred to a dedicated cryovial (Nalgene Nunc International, UK). Yeast cultures were stored at -80 °C.

2.2.3 Preparation of working stock cultures

1 mL of yeast culture from glycerol stock (Section 2.2.2) was transferred to 10 mL YPD media in a sterile universal bottle. The culture was incubated at 25 °C and 120 rpm for 48 hours in an orbital shaker (Braun Biotech, UK). 1 mL of the yeast culture was then transferred to 50 mL YPD in a sterile flask and grown at 25 °C and 120 rpm for 48 hours. After incubation, a loop-full of yeast culture was streaked onto YPD slopes or agar plates. Yeasts were then incubated at 25 °C in a static incubator (Sanyo, Japan) for 48 hours and stored at 4 °C as working stock cultures.

2.3 Determination of ale and lager type brewing yeast

2.3.1 Permissive growth temperature test

Yeast maximum growth temperature was determined according to a method adapted from Casey, *et al.* (1994). YPD agar plates (Section 2.2.1) were prepared prior to use. A loop-full of yeast culture was taken from an agar slope (Section 2.2.3) and streaked onto each of nine agar plates: three plates were then incubated at 25 °C, three at 34 °C and three at 37 °C, all for 7 days. Plates were examined every two days for microbial (yeast) growth. Temperatures at which growth was permitted was determined by the formation of colonies and subsequently used to determine brewing yeast type. Yeast capable of growing at 37 °C were considered to

be ale strains, while those which were not were considered to be lager strains according to accepted protocol (Barnett, *et al.*, 1983, Casey, *et al.*, 1994).

2.3.2 Rapid X- α -gal test

The melibiose analog 5-bromo-4-chloro-3-indolyl- α -D-galactopyranoside (X- α -gal) was used to differentiate ale and lager yeast based on the capacity of lager strains to cleave the X- α -gal molecule through the action of melibiase. Analysis was conducted according to the method of Box, *et al.* (2012). A stock solution of X- α -gal (Fisher Scientific, UK) was prepared by dissolving 62.5 mg X- α -gal in 10 mL 75 % (v/v in sterile RO water) 1, 2-propanediol (Fisher Scientific, UK). A loop-full of yeast culture from an agar slope (Section 2.2.3) was inoculated into 10 mL YPD media (Section 2.2.1) in a sterile universal bottle and incubated statically at 25 °C for 3 days. After incubation the supernatant was decanted and the yeast was re-suspended in 5 mL of sterile RO water. 100 μ L of yeast suspension was pipetted into a Eppendorf tube containing 10 μ L of X- α -gal solution. The mixture was incubated statically at 25 °C and examined after 30 min for colouration. A microfuge tube containing 100 μ L of yeast suspension without X-gal was also incubated simultaneously as a negative control. The appearance of a blue-green colour in samples tube indicated the presence of lager yeast. Yeast which did not yield a change in colour (sample remained cream or white) were considered to be ale yeast strains. Samples were re-checked after a further 24 hours and 7 days for confirmation purposes.

2.3.3 Growth on melibiose

The ability of yeast to utilise melibiose was analysed according to the method of Casey, *et al.* (1994). Yeast extract-peptone fermentation broth (0.45 % [w/v] yeast extract, 0.75 % [w/v] peptone and 0.005 % [w/v] bromothymol blue [pH indicator]) was prepared by autoclaving at 121 °C and 15 psi for 15 min. 2 mL aliquot of the media was dispensed into sterile Bijou

bottles containing an inverted Durham tube (50 × 6 mm). Subsequently, 1 mL of 12 % (w/v) sterile melibiose, 12 % (w/v) sterile glucose (positive control) or sterile RO water (negative control) was added to corresponding Bijou bottles. All chemicals were obtained from Fisher Scientific, UK.

A loop-full of yeast culture from an agar slope (Section 2.2.3) was inoculated into 10 mL YPD media (Section 2.2.1) in a sterile universal bottle and incubated at 25 °C for 48 hours. Once harvested, the cells were washed three times in sterile RO water before being re-suspended in 10 mL RO water. 100 µL of the cell suspension was then used to inoculate the test medium and control media, and samples were incubated at 25 °C for 7 days. The positive control (glucose fermentation broth) was analysed for gas production in Durham tubes and a change in colour from blue to yellow (conversion of bromothymol blue as a result of acid production), with neither gas production nor colour change in the negative control. The presence of a lager strain was indicated by both a colour change and gas production when grown on the test media supplemented with melibiose.

2.4 Determination of cell concentration and viability

Yeast cell concentration was determined based on a method adapted from Hatfield, *et al.* (1988). Cultures were diluted in RO water to reach a density of approximately 1.0×10^7 cells/mL and examined under microscope (Nikon, Japan) at 400× magnification using a haemocytometer (improved Neubauer counting chamber; Weber Scientific International Ltd, UK). 5 squares (top right, top left, bottom right, bottom left and middle squares) of yeast cells were counted within a 1 mm^2 ruled area (25 squares) × 0.1 mm thickness following a standard protocol: cells touching or resting on top and right boundary lines were enumerated, while cells touching or resting on bottom or left boundary lines were not counted; budding yeast cell were counted as one cell if the bud was less than one-half the size of the mother

cell. If the bud was equal to or greater than one-half the size of the mother cell, both cells were counted. At least 200 cells were counted to ensure statistical validity and cell density was calculated based on the formula below (Equation 2.1).

Cell viability was expressed as the percentage of viable cells within the total population (Equation 2.2). This value was estimated by brightfield staining using methylene blue (Section 2.4.1), or by fluorescent staining using either MgANS (Section 2.4.2) or oxonol (Section 2.4.3).

Equation 2.1 Calculation of cell concentration in yeast cell suspensions

$$\text{Cell concentration (cell/mL)} = \text{total cells in ruled area} \times \text{dilution factor (if any)} \times 5 \times 10^4$$

Where 10^4 represents the counting area of 10^{-4} cm^3

Equation 2.2 Calculation of cell viability in yeast cell suspensions

$$\text{Cell viability (\%)} = \frac{(\text{total cells}) - (\text{dead cells})}{\text{total cells}} \times 100 \%$$

2.4.1 Yeast viability by methylene blue staining

The methylene blue staining method traditionally employed in brewing laboratories was conducted according to the method of Pierce (1970). Methylene blue (Sigma, UK) was dissolved in 2 % (w/v) sodium citrate (Fisher Scientific, UK) solution to a final concentration of 0.01 % (w/v). Yeast cell suspensions were diluted with sterile RO water to reach a working solution of approximately 1.0×10^7 cells/mL (Section 2.4). A 0.5 mL aliquot of yeast suspension was then mixed with 0.5 mL methylene blue solution and gently agitated. The

solution was incubated for 5 min at room temperature and examined microscopically (Nikon, Japan) at a magnification of 400×. Cell viability was measured using a haemocytometer (improved Neubauer counting chamber; Weber Scientific International Ltd, UK) as described above (Section 2.4). Dark blue cells were counted as dead cells and those which remained unstained were considered to be viable cells. The number of live cells was expressed as a percentage of the total population (Equation 2.2).

2.4.2 Yeast viability by MgANS staining

Yeast viability determination using MgANS (1-Anilino-8-naphthalene-sulfonic acid) was performed according to the method of McCaig (1990). 0.3 g of MgANS (Sigma, UK) was dissolved in 2 mL of absolute ethanol (100 %, v/v; Fisher Scientific, UK) and diluted with 98 mL of sterile RO water to a final concentration of 0.3 % (w/v). Stock solutions were maintained at 4 °C in light-protected bottles for up to 6 months. Yeast cells were washed once in sterile RO water and re-suspended to a final concentration of approximately 1.0×10^7 cells/mL (Section 2.4). 0.5 mL of yeast suspension was mixed with 0.5 mL of MgANS solution and incubated for 5 min at room temperature in a dark environment. The solution was then examined under a fluorescence microscope (Nikon, Japan) at a magnification of 400× using a haemocytometer (improved Neubauer counting chamber; Weber Scientific International Ltd, UK). Non-viable cells were stained yellow-green and unstained cells were assumed to be viable. The number of live cells was expressed as a percentage of the total population (Equation 2.2).

2.4.3 Yeast viability by oxonol staining

Viability analysis using oxonol (bis-(1,3-dibutylbarbituric acid) trimethine oxonol; Dibac₄(3)) was conducted following the method of Lloyd and Dinsdale (2000). A stock solution was prepared by dissolving 1 mg of oxonol (Sigma, UK) in 1 mL of absolute ethanol (100 %, v/v;

Fisher Scientific, UK), and maintained at 4 °C in the dark. 10 µL of the stock solution was diluted in 1 mL RO water to produce a working solution of 10 µg/mL. Yeast cells were washed once in sterile RO water and re-suspended to a final concentration of approximately 1.0×10^7 cells/mL (Section 2.4). 900 µL of the yeast suspension was mixed with 100 µL of oxonol working solution and incubated for 5 min at room temperature in a dark environment. Cells were analysed under a fluorescence microscope (Nikon, Japan) at a magnification of 400× using a haemocytometer (improved Neubauer counting chamber; Weber Scientific International Ltd, UK). Non-viable cells were stained yellow-green and unstained cells were assumed to be viable. The number of live cells was expressed as a percentage of the total population (Equation 2.2).

2.5 Analysis of growth characteristics

2.5.1 Spot plate analysis

A spot plate technique was used to examine the effect of osmotic or ethanol stress on yeast growth characteristics. To determine osmotic stress tolerance, wort-sorbitol agar plates were prepared to contain 10 mL of 13 °P brewer's wort (Molson Coors Brewing Company, Burton-on-Trent, UK) and 10 mL of a mixture of 80 % (w/v) sorbitol (Fisher Scientific, UK) solution and sterile RO water to achieve final sorbitol concentrations of 0, 6, 12, 18, 24 and 30 % (w/v) (Table 2.2). Similarly, to determine ethanol stress tolerance, wort-ethanol agar plates were prepared containing 10 mL of 13 °P brewer's wort (Molson Coors Brewing Company, Burton-on-Trent, UK) supplemented with 10 mL of a mixture of 100 % (v/v) ethanol (Fisher Scientific, UK) and sterile RO water to achieve final ethanol concentrations of 0, 5, 10, 15 and 20 % (v/v) (Table 2.3). All the media was solidified with the addition of 1.2 % (w/v) agar (Fisher Scientific, UK).

Table 2.2 Preparation of wort-sorbitol plates for spot plate analysis

<i>Component</i>	<i>Wort-sorbitol plates (mL)</i>					
	<i>Wort+0 % sorbitol</i>	<i>Wort+6 % sorbitol</i>	<i>Wort+12 % sorbitol</i>	<i>Wort+18 % sorbitol</i>	<i>Wort+24 % sorbitol</i>	<i>Wort+30 % sorbitol</i>
13 °P brewer's wort	10	10	10	10	10	10
80 % (w/v) sorbitol	0	1.5	3.0	4.5	6	7.5
Sterile RO water	10	8.5	7	5.5	4	2.5
Total volume per plate	20	20	20	20	20	20

Table 2.3 Preparation of wort-ethanol plates for spot plate analysis

<i>Component</i>	<i>Wort-ethanol plates (mL)</i>				
	<i>Wort+0 % ethanol</i>	<i>Wort+5 % ethanol</i>	<i>Wort+10 % ethanol</i>	<i>Wort+15 % ethanol</i>	<i>Wort+20 % ethanol</i>
13 °P brewer's wort	10	10	10	10	10
100 % (v/v) ethanol	0	1	2	3	4
Sterile RO water	10	9	8	7	6
Total volume per plate	20	20	20	20	20

Starter yeast cultures were obtained by inoculating a loop-full of yeast from YPD slope (Section 2.2.3) into 10 mL YPD media (Section 2.2.1) and incubating in an orbital shaker (Sartorius, USA) at 25 °C and 120 rpm for 48 hours. 1 mL of the starter culture was then

transferred into a pre-sterilized 250 mL flask containing 100 mL of fresh YPD media. After incubation at 25 °C and 120 rpm for 48 hours, cell pellets were harvested by centrifugation (Eppendorf, UK) and washed twice with sterile RO water. A cell suspension was then prepared to obtain a final optimal density (OD) of 1.0 at a wavelength of 600 nm and diluted in 10^{-1} steps with sterile RO water to obtain four serial diluted samples. A volume of 3 μ L from each dilution was spotted onto the surface of wort-sorbitol or wort-ethanol agar plates prepared previously; each plate containing four yeast strains (Section 2.1). Plates were then incubated in a static incubator (Sanyo, Japan) at 25 °C for 5 days. Each test was performed in triplicate and the resulting growth was assessed visually and captured photographically using a gel imaging system (GelDoc, Bio-Rad Laboratories, USA) and VisionWorksLS software.

2.5.2 Growth curve analysis

In order to investigate growth characteristics under sorbitol-induced osmotic stress, kinetic growth curves of each brewing yeast strain were produced using a 96-well plate, with cell concentration determined using a TECAN automated micro-plate reader (Infinite® 200 PRO series, TECAN, UK). Wells were loaded to contain 49 μ L of 13 °P brewer's wort (Molson Coors Brewing Company, Burton-upon-Trent, UK) along with various volumes of 80 % (w/v) sorbitol solution in sterile RO water in order to achieve final sorbitol concentrations of 0, 6, 12, 18, 24 and 30 % (w/v) in a total volume of 46 μ L (Table 2.4). 3 μ L of yeast cell suspension ($OD_{600} = 1.0$; Section 2.5.1) was then added to initiate growth. Cells were incubated at 25 °C for 72 hours and readings were generated by measuring OD at 600 nm wavelength every 6 hours. Data was analysed and collected by Magellan™ Data Analysis Software (TECAN, UK).

Table 2.4 Preparation of wort-sorbitol for analysis of growth curve within 96-well plate

<i>Component</i>	<i>Wort-sorbitol media (μL)</i>					
	<i>Wort+0 % sorbitol</i>	<i>Wort+6 % sorbitol</i>	<i>Wort+12 % sorbitol</i>	<i>Wort+18 % sorbitol</i>	<i>Wort+24 % sorbitol</i>	<i>Wort+30 % sorbitol</i>
13 °P brewer's wort	49	49	49	49	49	49
80 % (w/v) sorbitol	0	7.4	14.7	22.1	29.4	36.8
Sterile RO water	46	38.6	31.3	23.9	16.6	9.2
Cell suspension	3	3	3	3	3	3
Total volume per well	98	98	98	98	98	98

2.6 Laboratory scale fermentation

2.6.1 Wort

Industrially produced wort (13 °P and 25 °P) was collected from Molson Coors Brewing Company (Burton-on-Trent, UK). High Gravity (HG; 18 °P) and Very High Gravity (VHG; 24 °P) worts were prepared by diluting the 25 °P un-hopped wort to the appropriate gravity using sterile RO water, whilst 13 °P hopped wort was used directly. Wort was dispensed into 1 L plastic containers (VWR, UK), sterilised (121 °C and 15 psi for 15 min) and frozen at -20 °C until required.

2.6.2 Wort supplementation and oxygenation

After thawing, worts were supplemented with 0.2 mg/L of zinc by the addition of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (Fisher Scientific, UK). In order to mimic industrial wort conditions as closely

as possible based on available lab facilities, HG (18 °P) and VHG (24 °P) worts were sparged with oxygen for 3 hours at a flow rate of 0.5 L/min in a pre-autoclaved 5 L container (Figure 2.1). The worts were ultimately expected to be saturated with oxygen and the resultant dissolved oxygen (DO) levels were estimated to be approximately 38 and 35 ppm, respectively, established according to the oxygen solubility in wort at each specific gravity and oxygenation temperature (Boulton & Quain, 2001). The standard gravity (13 °P) wort was aerated with stirring (350 rpm) for 24 hours and was expected to be air-saturated with a DO level of approximately 9 ppm, again based on solubility of air in wort at the aeration temperature and the wort gravity (Boulton & Quain, 2001).

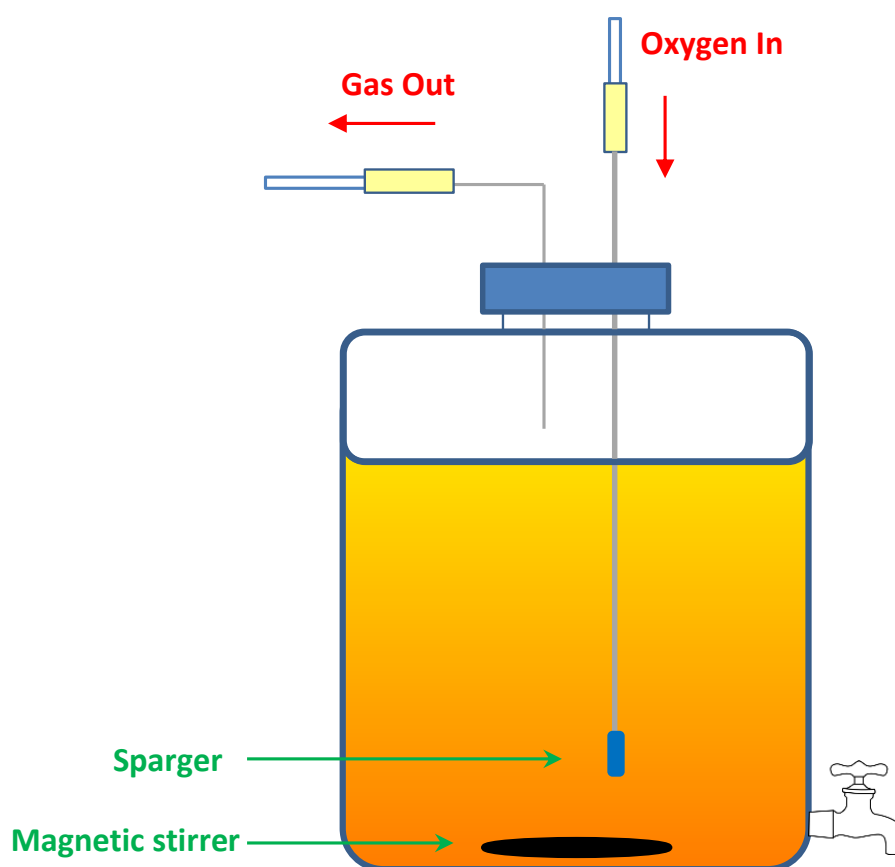


Figure 2.1 Wort oxygenation system (5L) used for wort preparation

2.6.3 Mini fermentation system

Mini laboratory scale fermentations were performed in glass hypo-vials using a method adapted from Quain, *et al.* (1985) and Powell, *et al.* (2003). 150 mL hypo-vials (International Bottle Company, UK) containing a magnetic stirrer (Figure 2.2) were autoclaved at 121°C and 15 psi for 15 min prior to use. A 100 mL volume of brewer's wort (Section 2.6.1) was aliquoted into each hypo-vial, pitched with yeast (Section 2.6.5), and the vials were sealed using rubber septa and metal crimps (VWR, UK). A gas outlet port was established using a sterile needle and Durham tube connected via a section of silicone tubing with a narrow slit in its structure known as a Bunsen valve (Figure 2.2). Homogeneity was ensured by agitation (350 rpm) using a flatbed 15-place magnetic stirring plate. Fermentations were performed in a 15 °C incubator (Sanyo, Japan) in accordance with a typical lager brewing temperature profile (Figure 2.3).

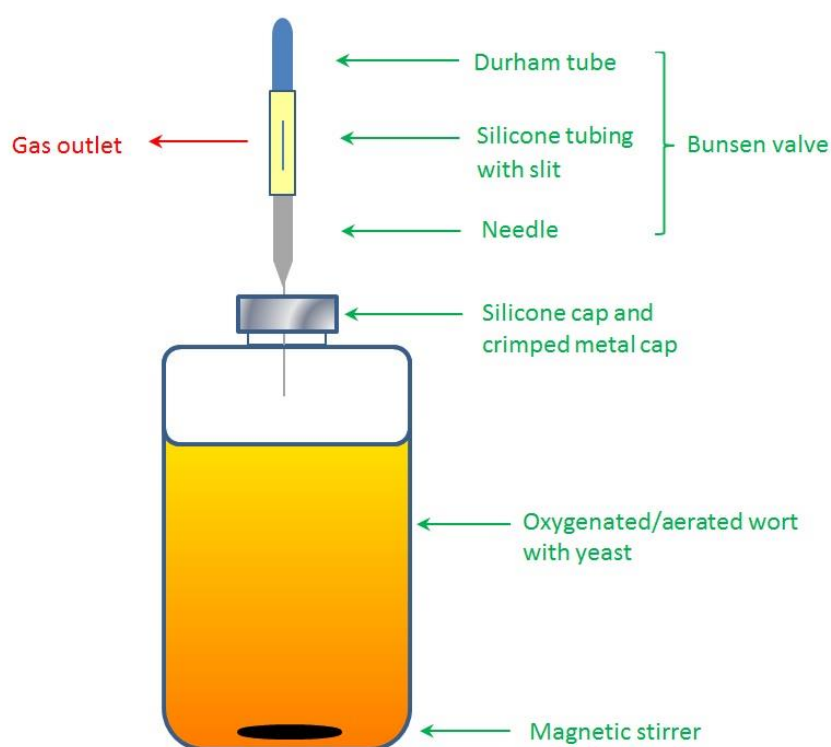


Figure 2.2 Mini fermenter (100 mL) used for fermentation analysis



Figure 2.3 Laboratory scale fermentation performed in the study

2.6.4 Yeast propagation

Yeast propagation was conducted with the aim of modelling the process which occurs in breweries. However, although this would normally be completed using wort, YPD media was used to eliminate potential variation caused by different wort composition, leading to a standard and more reproducible starting yeast culture. Three successive cultures of increasing volume were carried out in order to achieve the required cell numbers. A starter culture was obtained by inoculating yeast strains from YPD slope (Section 2.2.3) into 10 mL YPD media (Section 2.2.1) and incubating in a shaker (Braun Biotech, UK) for 48 hours at 25 °C and 120 rpm. 1 mL of the yeast culture was then transferred to a pre-sterilized 250 mL conical flask containing 100 mL YPD media and yeast was grown at 25 °C and 120 rpm for 48 hours. Finally, 8 mL of the culture was transferred to a pre-sterilized 2 L conical flask containing 800 mL YPD media. The yeast culture was shaken at 120 rpm and 25 °C for 48 hours prior to pitching.

2.6.5 Yeast pitching

To obtain the pitching yeast inoculum, 800 mL of yeast culture from propagation (Section 2.6.4) was centrifuged at 4,000 rpm for 5 min at 4 °C (Beckman, UK) using pre-weighed sterile centrifuge pots (Fisher Scientific, UK). The pellet was weighed and re-suspended in an equal volume of sterile RO water to obtain 50 % (w/w) slurry. Viable cell density was determined using methylene blue staining (Section 2.4.1) and subsequently yeast slurry was pitched into fermentation vessels (Section 2.6.3) to achieve the required number of viable cells as described in Table 2.5. These values were selected to compare the ‘standard’ pitching rate (1.5×10^7 viable cells/mL. irrespective of initial wort gravity) to the adjusted pitching rate (1.0×10^6 viable cells/mL per degree Plato), and the latter was generally regarded as ‘rule of thumb’ in brewing practice (Stewart, 2009). It should be noted that the experimental programme (Table 2.5) was conducted in sequence.

Table 2.5 Properties and abbreviation of five experimental conditions

<i>Test condition</i>	<i>Wort gravity (°P)</i>	<i>Pitching rate (Viable cells per mL)</i>
13P15M	13	1.5×10^7
18P15M	18	1.5×10^7
18P18M	18	1.8×10^7
24P15M	24	1.5×10^7
24P24M	24	2.4×10^7

2.6.6 Fermentation analysis and sampling

Fermentation progression was monitored by measuring wort utilisation in terms of weight loss of mini-fermenters over time. This was assessed using a Sartorius balance (Sartorius, UK) by subtracting vessel weight at each sampling point from the vessel weight at the beginning of fermentation. Fermentation was conducted for 120 hours, and for each set of fermentation parameters, destructive samples were taken at approximately 0, 3, 15, 24, 48, 65, 90 and 120 hours post-pitching. At each time point, three fermentation vessels (Section 2.6.3) were removed and immediately stored on ice for analysis. Cell density and viability were determined using methylene blue staining (Section 2.4.1). Cell fractions and wort/beer samples were isolated by centrifugation at 4,000 rpm for 5 min (Eppendorf, UK). Wort and beer samples were subsequently transferred to a fresh centrifuge tube and stored at -20 °C, whilst cell pellets were washed twice in sterile RO water and stored at -80 °C prior to analysis (Sections 2.7-2.8).

2.7 Analysis of wort and beer samples

2.7.1 Determination of wort gravity in wort and beer samples

Fermentation samples (Section 2.6.6) were thawed and analysed using an AlcoLyzer Plus beer system incorporating a DMA 4500 density meter (Anton Paar Ltd., UK). Gravity was measured using an oscillating U-tube and expressed in °P. Prior to analysis, high purified water (Millipore Corporation, USA) was used to calibrate the instrument until the data output was within the manufacturer's specified range.

2.7.2 Determination of sugar content in wort and beer samples

Wort sugars were analysed using high-performance liquid chromatography (HPLC) based on the method of Gibson, *et al.* (2008). External standards of each sugar were prepared in

gradient concentrations as outlined in Table 2.6. Stock solutions of each sugar were prepared in a volume of 10 mL (STD 1), which was sequentially diluted to produce STD 2-5 using high purified water (Millipore Corporation, USA). 100 mg/mL melezitose (Fisher Scientific, UK) was prepared as an internal standard (IS). 1 mL of wort or beer sample (Section 2.6.6) was mixed with 100 μ L of IS in a 1.5 mL microfuge tube. The mixture was subsequently passed through a solid phase extraction cartridge (strata-X 33 μ m Polymeric Reversed Phase 30 mg/mL cartridge, Phenomenex, UK) previously conditioned with 1 mL methanol (Fisher Scientific, UK) and equilibrated with 1 mL sterile RO water. The first five drops of the sample to pass through the cartridge were discarded; the next 1 mL of the sample was collected in a 2 mL screw neck vial (VWR, UK) for analysis.

Samples were placed in an automatic sampler set to follow a random running order. The purpose of the random running order was to ensure any systematic variation within the analysis apparatus were not biased towards particular time points. 10 μ L of each sample was injected onto an HPLC column (250 mm \times 2.0 mm internal diameter, Luna NH₂ column with 5 μ m particle size, Phenomenex, UK) preheated to 40 °C. The samples were eluted using acetonitrile:water (80:20, v/v) at a flow rate of 0.7 mL/min. Peak signals were detected by a refractive index detector (RI 2031 plus, JASCO, Japan) and analysed using AZUR chromatography data system (Datalys, France) in triplicate. Peaks were manually assigned according to the expected retention time of the sugars (Table 2.6) established in the lab. The peak area of each compound was normalised by obtaining the ratio of the target compound to the internal standard. Data obtained from the external standards was then plotted against their known concentrations in order to determine the quantifying factors for unknown samples. If the response was linear as expected, the gradient value of the graph was subsequently used as the factor to calculate sugar quantity in the samples (Equation 2.3).

Table 2.6 Concentrations and retention times of standard sugars used in HPLC analysis

<i>Compounds</i>	<i>Concentrations (g/L)</i>					<i>Expected Retention Time (min)</i>
	<i>STD 1</i>	<i>STD 2</i>	<i>STD 3</i>	<i>STD 4</i>	<i>STD 5</i>	
<i>Fructose</i>	10	5	2.5	1.25	0.675	2.60 ± 0.05
<i>Glucose</i>	30	15	7.5	3.75	1.875	3.25 ± 0.04
<i>Sucrose</i>	12	6	3	1.5	0.75	4.70 ± 0.07
<i>Maltose</i>	120	60	30	15	7.5	5.42 ± 0.06
<i>Melzitose (IS)</i>	10	10	10	10	10	9.00 ± 0.08
<i>Maltotriose</i>	60	30	15	7.5	3.75	10.05 ± 0.09

Equation 2.3 Calculation of sugar content in wort by HPLC

$$\text{Target compound (g/L)} = \frac{\text{Peak area of target compound}}{\text{Peak area of internal standard}} \times \text{Gradient}$$

2.7.3 Determination of ethanol and glycerol in wort and beer samples

Quantification of ethanol and glycerol in samples was conducted by HPLC analysis using a 300 × 7.8 mm internal diameter, Rezex ROA organic acid column (Phenomenex, UK) under ambient temperature. Standard solutions were prepared as outlined in Table 2.7. 1 mL of the standard or sample (Section 2.6.6) was pipetted into a 2 mL screw neck vial (VWR, UK) and placed in an automatic sampler set to follow a random running order as described above (Section 2.7.2). 10 µL of the sample was eluted using 2.5 mM H₂SO₄ solution (Fisher Scientific, UK) at a flow rate of 0.5 mL/min into a refractive index detector (RI 2031 plus, JASCO, Japan). Peak signals were analysed using AZUR chromatography data system (Datalys, France), where peaks were automatically assigned according to the expected

retention time established in the lab (Table 2.7). The peak areas of standards were then plotted against their known concentrations in order to determine the quantifying factors for unknown samples. If the response was linear as expected, the gradient value of the graph was subsequently used as the factor to quantify the compounds in the samples (Equation 2.4).

Table 2.7 Concentrations and retention times of standard glycerol and ethanol used in HPLC analysis

<i>Compounds</i>	<i>Concentrations (g/L)</i>					<i>Expect Retention Time (min)</i>
	<i>STD 1</i>	<i>STD 2</i>	<i>STD 3</i>	<i>STD 4</i>	<i>STD 5</i>	
Glycerol	10	5	2.5	1.25	0.625	16.71 ± 0.09
Ethanol	100	50	25	12.5	6.25	24.05 ± 0.08

Equation 2.4 Calculation of glycerol and ethanol content in wort by HPLC

$$\text{Target compound (g/L)} = \text{Peak area of target compound} \times \text{Gradient}$$

2.7.4 Determination of flavour compounds in wort and beer samples

Flavour compounds within samples (Section 2.6.6) were detected using headspace gas chromatography-mass spectrometry (GC-MS) according to the method of Ashraf, *et al.* (2010). A standard stock solution of each compound of interest (Table 2.8) was prepared to a concentration of 50 mg/L in RO water. The stock solution was then serially diluted at a ratio of 1:2, 1:5, 1:10 and 1:100 to achieve a final concentration of 25, 10, 5 and 0.5 mg/L, respectively. In each instance, 5 mL of the beer sample (Section 2.6.6) or the standard

solution was transferred into a 20 mL headspace glass vial (75.5×22.5 mm, DIN crimp neck, round bottom; Fisher Scientific, UK) containing 100 μ L of 2-butanol solution (125 μ L 2-Butanol in 100 mL 100 % methanol; Fisher Scientific, UK), served as Internal Standard (IS). Vials were sealed using magnetic crimps (8 mm central hole, PTFE silicon septa; Fisher Scientific, UK) with pre-fitted septum (Fisher Scientific, UK) and placed in an automatic sampler following a random running order as described previously (Section 2.7.2).

Samples were heated to 40 °C for 20 min before an injection of 250 μ L of the headspace using a gas-tight syringe (CTC Analytics AG, Switzerland). Analysis was performed by a Trace GC ULTRA (Thermo Scientific, USA) by applying a splitless mode using a Combi PAL autosampler (CTC Analytics, Switzerland), equipped with a ZB Wax column ($30 \text{ m} \times 0.25 \text{ mm i.d. } 1 \mu\text{m}$ film thickness (Phenomenex, UK). The temperature programme for the oven was: 40 °C for 5 min, 10 °C/min to 100 °C and then 100 °C for 2.5 min. Helium was used as carrier gas at a flow rate of 1.5 mL/min. Chromatograms were recorded using a DSQ mass spectrometer (Thermo Fisher Scientific, UK) operated in selected ion mode (Table 2.8) and analysed using Xcalibur software (Thermo Fisher Scientific, UK). The peaks were assigned manually by subtracting the baseline signal from the peak height. Peak areas of the standards were then plotted against their known concentrations in order to determine the quantifying factors. If the response was linear as expected, the gradient value of the graph was subsequently used as the factor to quantify the flavour compounds in the samples (Equation 2.5).

Table 2.8 Compounds in the calibrate solution used in headspace GC-MS analysis

<i>Compounds</i>	<i>Expect Retention Time (min)</i>	<i>detected ions (m/z)</i>
Acetaldehyde	2.22	44
Ethyl acetate	5.71	43
Ethyl propionate	7.86	56
Isobutyl acetate	9.53	73
2-Butanol (IS)	9.8	59
Ethyl butyrate	10.09	71
n-Propanol	10.56	59
Isobutanol	11.16	43
Isoamyl acetate	11.77	70
1-Butanol	12.12	56
2-Methyl-1-butanol	13.42	57
3-Methyl-1-butanol	13.43	42

Equation 2.5 Calculation of flavour contents in beers by headspace GC-MS analysis

$$\text{Target compound (g/L)} = \frac{\text{Peak area of target compound}}{\text{Peak area of internal standard}} \times \text{Gradient}$$

2.7.5 Determination of pH in wort and beer samples

Fermentation samples (Section 2.6.6) were thawed, if required, and pH was measured using a pH meter (Mettler Toledo, UK). The pH meter was calibrated using standard solutions of pH 4.00 and 7.00 (Fisher Scientific, UK) prior to use. All data reflects the analysis of triplicate independent samples.

2.8 Analysis of yeast samples

2.8.1 Determination of trehalose and glycogen in yeast cell fractions

Intracellular glycogen and trehalose were quantified using a method based on that described previously (Parrou & Francois, 1997). 1.0×10^9 total cells were suspended in 250 μ L of 0.25 M Na₂CO₃ (Fisher Scientific, UK) and incubated in a 95 °C water bath (Clifton, UK) for 2 hours with occasional mixing. After cooling, 600 μ L of 0.2 M sodium acetate (Fisher Scientific, UK) and 150 μ L of 1 M acetic acid (Fisher Scientific, UK) were added to the suspension. Subsequently 500 μ L of the mixture was transferred to a microfuge tube and incubated at 57 °C for 10 hours in the presence of 100 μ g of freshly prepared amyloglucosidase (Sigma, UK). The other 500 μ L of the mixture was incubated at 37 °C for 10 hours in the presence of 3 mU of freshly prepared trehalase (Sigma, UK). After incubation, samples were centrifuged at 13,000 rpm for 2 min and the liberated glucose in suspension was determined using a glucose assay kit (Megazyme, Ireland). 0.1 mL of the supernatant, standard glucose (1.0 mg/mL D-glucose) or blank (sterile RO water) was pipetted into a test tube containing 3.0 mL of GOPOD (glucose oxidase/peroxidase) reagent (Table 2.9), respectively. The tubes were incubated in a 45 °C water bath (Clifton, UK) for 20 min. The absorbance of the solution was read at 510 nm against a reagent blank to obtain A(sample) and A(standard) using a spectrophotometer (Jenway 7315, Camlab, UK). The glucose content

was quantified accordingly (Equation 2.6). Consequently, the concentration of trehalose or glycogen was expressed in units of μg glucose per 10^8 cells.

The principle of the glucose assay was as follows: glucose in suspension was oxidised to form D-gluconate and H_2O_2 , catalysed by glucose oxidase in the presence of oxygen and H_2O . The resulting hydrogen dioxide, along with p-hydroxybenzoic acid and 4-aminoantipyrine was then converted by peroxidase to form quinoneimine dye, which was recorded by absorbance at 510 nm.

Table 2.9 Preparation of solutions used in glucose assay (Megazyme, Ireland)

	<i>Standard (mL)</i>	<i>Blank (mL)</i>	<i>Sample (mL)</i>
<i>GOPOD Reagent</i>	3.0	3.0	3.0
<i>Standard</i>	0.1	-	-
<i>Blank</i>	-	0.1	-
<i>Sample</i>	-	-	0.1

Equation 2.6 Calculation of glucose content derived from intracellular glycogen and trehalose using a glucose assay kit

$$\text{Glucose (g/0.1 mL)} = \frac{A(\text{sample})}{A(\text{standard})} \times 100$$

2.8.2 Determination of glycerol content in yeast cell fractions

Intracellular glycerol was quantified according to the method of Hounsa, *et al.* (1998). 1.0×10^9 total cells were suspended for 10 min in 1 mL boiling Tris-HCl (0.5 M Tris-base adjusted to pH 7.0 with 0.5 M HCl and sterilised by autoclaving at 121 °C and 15 psi for 15 min; chemicals supplied by Sigma, UK). The supernatant was harvested by centrifugation at 4,000 rpm for 10 min (Eppendorf, UK) and analysed using a glycerol assay kit (Megazyme, Ireland). Samples were prepared in cuvettes as described in Table 2.10. Absorbance before and after the addition of solution 4 was measured spectrophotometrically at a wavelength of 340 nm (Jenway, 7315, Camlab, UK), and recorded as A1 and A2, respectively. The absorbance difference (A2-A1) was determined for both blank and sample, and ΔA (glycerol) was obtained by subtracting the absorbance difference of the blank from the absorbance difference of the sample. The concentration of glycerol was calculated accordingly (Equation 2.7) and consequently expressed in units of μg glycerol per 10^8 cells. All experiments were conducted in triplicate.

The principle of the assay was as follows: glycerol in suspension was phosphorylated to form L-glycerol-3-phosphate, catalysed by glycerokinase in the presence of adenosine-5'-triphosphate (ATP), which was reduced to adenosine-5'-diphosphate (ADP). The resulting ADP was reconverted into ATP by phosphoenolpyruvate (PEP) and pyruvate kinase (PK) with the formation of pyruvate. Pyruvate was reduced to L-lactate in the presence of L-lactate dehydrogenase (L-LDH) by reduced nicotinamide-adenine dinucleotide (NADH) with the resultant oxidation of NADH to NAD^+ . The amount of NADH oxidised is stoichiometric to the amount of glycerol, and this was recorded on the basis of its light absorption at 340nm.

Table 2.10 Preparation of solutions used in glycerol assay (Megazyme, Ireland)

	<i>Blank (mL)</i>	<i>Sample (mL)</i>
<i>Sterile RO water</i>	1.6	1.5
<i>Sample</i>	-	0.1
<i>Solution 1</i>	0.20	0.20
<i>Solution 2</i>	0.20	0.20
<i>Solution 3</i>	0.02	0.02
<i>Solution 4</i>	0.02	0.02

Equation 2.7 Calculation of intracellular glycerol using a glycerol assay kit

$$\text{Glycerol (g/L)} = 0.2982 \times \Delta A (\text{glycerol}) \times \text{dillution factor (if any)}$$

2.8.3 Determination of yeast dry weight

Yeast dry weight was determined by a method based on filtration and desiccation. Filter paper (VWR, UK) was dried on an aluminium tray (VWR, UK) at 55 °C in an oven overnight and then kept in a desiccator prior to use. The dried filter paper and tray were weighed (A) and then positioned on top of a vacuum filtration apparatus (VWR, UK). 10 mL of cell suspension at the start (0 hour) or the end (120 hour) of each set of fermentation (Section 2.6.6) was pipetted onto the filter paper and a vacuum was applied to remove all trace of supernatant. The filter paper was transferred back to the same tray and thereafter dried at 55 °C in an oven until a constant weight was achieved (B). The dry weight was calculated using Equation 2.8.

Equation 2.8 Calculation of yeast dry weight of cell pellets

$$\text{Dry weight (g/L)} = \frac{B - A}{\text{sample volume (mL)}} \times 1000$$

Where A = weight of dried filter and tray (g), B = weight of dried filter, dried residue and tray (g)

2.9 Estimation of yeast carbon partitioning

In order to estimate yeast carbon partitioning under different fermentation conditions, certain assumptions were made based on the observations of Cutaia (2007): (I) carbon conversion by assimilation of non-carbohydrate materials from wort, such as free amino nitrogen, was considered negligible; (II) carbon conversion to fermentation products other than ethanol, carbon dioxide, glycerol, yeast biomass, glycogen, trehalose, higher alcohols and esters, was considered to be negligible, such as organic acids. Thereafter, equations for this calculation were derived (Section 2.9.1) and subsequently, yeast carbon distribution was obtained and expressed as a percentage of the total carbon input (Section 2.9.2).

2.9.1 Derivation of equations

Estimation of carbon content was based on analysis of key products as described previously (Sections 2.7-2.8). Equation 2.9 was derived from carbon conservation within each compound and mass balance consideration (Table 2.11). Corresponding carbon content was quantified from the fermentable carbohydrate profile of the initial and residual worts (Section 2.7.2), the concentration of ethanol (Section 2.7.3), carbon dioxide (Section 2.6.6) and glycerol (Section 2.7.3), as well as higher alcohols and esters (Section 2.7.4) in the resulting beers. In addition, the carbon concentrations in trehalose and glycogen (Section 2.8.1) was quantified from liberated glucose contents using Equation 2.10.

Table 2.11 Molecular weight and carbon percentage of carbon compounds in samples (*ratio of carbon in the compound)

<i>Compounds</i>	<i>Molecular weight</i>	<i>Molecular weight of carbon</i>	<i>Carbon(%)[*] (Rc)</i>
<i>Fructose</i>	180.1	72.0	40.0
<i>Glucose</i>	180.1	72.0	40.0
<i>Sucrose</i>	342.0	144.0	42.1
<i>Maltose</i>	342.0	144.0	42.1
<i>Maltotriose</i>	504.4	216.0	42.8
<i>Ethanol</i>	46.1	24.0	52.1
<i>Carbon dioxide</i>	44.0	12.0	27.3
<i>Glycerol</i>	92.1	36.0	39.1
<i>Ethyl acetate</i>	88.1	48.0	54.5
<i>Ethyl propionate</i>	102.1	60.0	58.7
<i>Ethyl butyrate</i>	116.2	72.0	62.0
<i>Isobutanol</i>	74.1	48	64.8
<i>Isoamyl acetate</i>	130.2	84.0	64.5
<i>2-methyl-1-butanol</i>	88.2	60.0	68.1
<i>Ethyl hexanoate</i>	144.2	96.0	66.6

Equation 2.9 Calculation of carbon content in wort and beer samples

$$C1 (g/L) = Cg \times Rc$$

Where Cg is the concentration for a given compound (g/L), Rc is the ratio of carbon in the compound (Table 2.11)

Equation 2.10 Calculation of carbon contents in trehalose or glycogen

$$C2 (g/L) = \frac{Nt}{10^8} \times Ci \times 10^{-5} \times 40.0 \%$$

Where Nt is the total cell numbers, Ci is the concentration of trehalose or glycogen in the form of glucose ($\mu\text{g}/10^8\text{cells}$), 10^{-5} is an unit conversion factor and 40.0 % represents carbon percentage in glucose.

2.9.2 Total carbon input and carbon partitioning

Based on previous assumptions (Section 2.9), the total carbon input was considered to be the sum of the carbon content of wort carbohydrates consumed during each set of fermentation. This was calculated from the concentration of each wort carbohydrate (fructose, glucose, sucrose, maltose and maltotriose) in the original wort and the final beer using Equation 2.9 (Section 2.9.1). The total carbon input was then used to quantify carbon investment (percent of total carbon input) in each metabolite, including ethanol, carbon dioxide, glycerol, higher alcohols and esters, as well as trehalose and glycogen and expressed as percent of the total carbon input.

Apart from the allocated carbon proportion of total carbon input, the ‘un-allocated’ percentage of carbon was attributed to yeast biomass, most likely as an artefact of cell maintenance, growth and division. This assumption was made based on the observations of Cutaia (2007). Alternatively, the increase of yeast dry weight at the end of fermentations was obtained (Section 2.8.3) and the carbon concentration in the yeast biomass was calculated based on a carbon content of 48 % in dry wine yeast (Rosen, 1989) and expressed in percent of the total carbon input. The two estimations described above were used to ascertain the carbon distribution in yeast biomass throughout fermentations.

2.10 Assessment of osmolality using a micro-osmometer

Osmolality is defined as the number of milliosmoles of solute particles per kilogram of pure solvent and expressed as mOsm/kg (Erstad, 2003) and was used to express the osmotic potential of a solution. Osmolality values were determined using a micro-osmometer (Figure 2.4; Advanced Instrument, USA) based on freezing point depression. Freezing point depression describes the phenomenon whereby the freezing point of a liquid (a solvent) is depressed when another compound (solute) is added, meaning that a solution will have a lower freezing point than a pure solvent. Therefore, when a solute is dissolved in a pure solvent, the colligative properties of the solution change in direct proportion to the solute concentration (Koumantakis & Wyndham, 1989).

The micro-osmometer was calibrated prior to use according to the manufacturer's operating instructions. Subsequently, approximately 20 μ L of sample was taken using a specific sample tip (Advanced Instrument, USA) and the osmolality was measured automatically via a probe used to determine freezing point depression. To prevent contamination, the probe was cleaned and dried using a chamber cleaner (Advanced Instrument, USA) between each measurement.



Figure 2.4 The micro-osmometer instrument (Advanced Instrument, USA)

2.10.1 Determination of osmolality in wort and beer samples

Fermentation samples (Section 2.6.6) were thawed, if required, and the osmolality values were determined using the micro-osmometer (Figure 2.4) as described above.

2.10.2 Determination of osmolality in standard wort, ethanol and glycerol

In order to investigate the contribution of potential compounds to the changing osmolality during fermentation (Section 2.6), the 24 °P original wort (Section 2.6.1) was diluted to 75, 50 and 25 % (v/v) using RO water, respectively and the osmolality of the dilutions were determined using the micro-osmometer (Figure 2.4). In addition, osmolality values of ethanol solutions (10, 20, 30, 40, 50, 60, 70, 80 and 90 g/L) and glycerol solutions (1, 2, 3, 4 and 5 g/L) were also measured. RO water was used as a control (0 mOsm/kg). It should be noted that the concentrations used here were selected to cover the range of individual compounds observed throughout standard, HG and VHG fermentations, as reported in Chapter 4.

2.10.3 Induction of extracellular osmolality using sorbitol

In order to mimic extracellular osmolality encountered during fermentations, sorbitol solutions (10, 20, 30, 40 and 50 %, w/v) were prepared in order to achieve a series of environments with osmolality values ranging from approximately 563-3010 mOsm/kg (Table 2.12). These were used to conduct fermentation-based analyses, alongside sterile RO water (0 mOsm/kg) as a baseline control (Table 2.12). Media solutions were sterilised by autoclaving at 121 °C and 15 psi for 15 min. Yeast propagation was conducted as described previously (Section 2.6.4). Exponential and stationary phase cells were harvested and washed twice in sterile RO water. The cells were then re-suspended in 100 mL of either sterile RO water or sorbitol solution to achieve a concentration of 5.0×10^8 cells/mL, and incubated on an orbital shaker (Braun Biotech, UK) at 15 °C and 120 rpm for 48 hours prior to analysis. Consequently, yeast osmotic stress response was determined based on analysis of cell viability (Section 2.4), vitality (Section 2.11), production of cellular protectants (Section 2.8), profile of membrane fluidity (Section 2.12) and genomic DNA fingerprinting (Section 2.13). All experiments were conducted in triplicate.

Table 2.12 Osmolality values of prepared sorbitol solutions. Values represent the mean \pm standard derivation of triplicate samples.

<i>Sorbitol concentration (% w/v)</i>	<i>Osmolality (mOsm/kg)</i>
0	0
10	563 \pm 18
20	1125 \pm 12
30	1688 \pm 16
40	2250 \pm 20
50	2813 \pm 25

2.11 Assessment of yeast vitality using acidification power test

Yeast vitality was determined using the acidification power test according to Siddique and Smart (2000). This assay was performed to assess yeast activity in terms of sugar utilisation via the measurement of proton efflux in cell populations. The assay contained two calculated components, water acidification power (WAP) and glucose acidification power (GAP), quantified before and after the addition of glucose. The determination of WAP and GAP allows the calculation of net glucose induced proton efflux (GIPE), which reflects the net efflux of proton across the cell membrane induced only by glucose utilisation.

2.11.1 Water acidification power test

Yeast cells (Section 2.10.3) were harvested by centrifugation (Eppendorf, UK) and washed three times using sterile RO water. Yeast suspensions were then prepared by re-suspending cell pellets in sterile RO water to reach a final cell concentration of 5.0×10^8 cells/mL. A pre-calibrated pH probe (Mettler Toledo, UK) was placed into a sterile universal bottle, containing 19 mL sterile RO water and a magnetic flea, on a magnetic stirrer plate. Once the pH of water was equilibrated, 1 mL of the cell suspension was added followed by immediate measurement of an initial pH, defined as WAP0. After 10 min, 5 mL of sterile RO water was added and pH measurement continued for a further 10 min. After 20 min, the pH value was recorded, defined as WAP20 and consequently WAP was calculated according to Equation 2.11 below.

Equation 2.11 Calculation of water acidification power (WAP)

$$WAP = WAP0 - WAP20$$

2.11.2 Glucose acidification power test

Cell suspensions (5.0×10^8 cells/mL) were prepared as described above (Section 2.11.1). The pre-calibrated pH probe was then placed into a sterile universal bottle, containing 19 mL sterile RO water and a magnetic flea, on a magnetic stirrer plate. Once the pH of water was equilibrated, 1 mL of the cell suspension was added followed by an immediate pH measurement, designated GAP0. The pH value was monitored at 2 min intervals. After 10 min, 5 mL of sterile 20.2 % (w/v) glucose solution (Fisher Scientific, UK) was immediately added and pH measurement continued for a further 10 min. After 20 min, the pH value was noted as GAP20. The GAP was calculated (Equation 2.12) and consequently GIPE was calculated according to Equation 2.13.

Equation 2.12 Calculation of glucose acidification power (GAP)

$$GAP = GAP0 - GAP20$$

Equation 2.13 Calculation of net glucose induced proton efflux (GIPE)

$$GIPE = GAP - WAP$$

2.12 Assessment of membrane fluidity

Yeast membrane fluidity was determined by fluorescent staining using laurdan, based on the methods of Learmonth and Gratton (2002) and Walker, *et al.* (2006). The membrane probe laurdan (6-lauroyl-2-dimethylamino naphthalene) is known to be sensitive to environmental polarity (Weber & Farris, 1979, Parasassi, *et al.*, 1990, Parasassi, *et al.*, 1991) and localises at the hydrophilic-hydrophobic interface of the cell membrane (Chong & Kao, 1990). Laurdan

relates to membrane fluidity by creating different maxima and shape of emission spectra in the liquid-crystalline and gel phases of membrane, due to differences in polarity and the amount of dipolar relaxation (Parasassi & Gratton, 1995). The Generalized Polarization (GP) value, derived from laurdan fluorescence, was thus used as an index of membrane fluidity.

Prior to analysis, 5 mM of laurdan stock was prepared by the addition of 5 mg of laurdan (Molecular Probes, Invitrogen, USA) to 2.83 mL of absolute ethanol (100 %, v/v; Fisher Scientific, UK) in a light protected glass tube. The mixture was vortexed and warmed gently (approximately 30 °C) to dissolve. The resultant solution was stored at room temperature.

2.12.1 Cell labelling and determination of generalized polarization

Cell pellets (Section 2.10.3) were harvested by centrifugation (Eppendorf, UK), washed once in sterile RO water and re-suspended in sterile RO water to achieve an OD of 0.1 at a wavelength of 600 nm. Cell labelling was conducted by mixing laurdan stock with the cell suspension to achieve a final concentration of 5 μ M. The mixture was incubated in the dark at room temperature for 1 hour and 200 μ L of the solution was pipetted into a 96-well plate (Nunc, Thermo Fisher Scientific, UK). Fluorescence was measured at an excitation wavelength (350 nm) and two emission wavelengths (440 nm and 490 nm) via a Varioskan Flash micro-plate reader (Thermo Fisher Scientific, UK). The fluorescence intensity at wavelengths 440 nm and 490 nm, corresponding to the emission maxima of laurdan in the gel and in the liquid crystalline phases, respectively, was used for GP calculation (Equation 2.14) as an index of membrane fluidity. Theoretically GP value ranges from -1 to $+1$ and are inversely related to membrane fluidity, where high GP value indicates low membrane fluidity (Parasassi, *et al.*, 1990).

Equation 2.14 Calculation of GP value as an index of membrane fluidity

$$GP = \frac{I_{440} - I_{490}}{I_{440} + I_{490}}$$

Where I_{440} and I_{490} indicate relative fluorescence intensities at wavelengths representing gel (440 nm) and liquid crystalline (490 nm) phases of bilayer systems, respectively.

2.13 Analysis of yeast DNA

2.13.1 Extraction of genomic DNA

Genomic DNA was extracted according to a method described by Powell and Diacetis (2007). Yeast cells (Section 2.10.3) were harvested and suspended in 660 μ L 50TE-SDS buffer. 50TE-SDS buffer comprised a mixture of 200 mL of 50TE buffer (7.44 g/L EDTA, 6.06 g/L TRIS, adjusted to pH 7.5 with 1 M HCl and sterilised by autoclaving at 121 °C and 15 psi for 15 min) and 20 mL of a 10 % SDS solution (Sigma, UK), filter sterilised by 0.45 μ M filter (Millipore, UK). Suspended cells were vortexed and incubated in a 65 °C water bath (Clifton, UK) for 10 min. Subsequently 340 μ L of potassium acetate (5 M) was added and samples were incubated at 4 °C for approximately 15 min. The sediment was removed by centrifugation for 10 min at 13,000 rpm and 600 μ L of supernatant was then transferred into a fresh Eppendorf tube containing an equal quantity of isopropanol (Fisher Scientific, UK). Samples were maintained at room temperature for 10 min before centrifugation at 10,000 rpm for 10 min. The aqueous phase was discarded and DNA pellets were washed once using 100 μ L of 95 % (v/v) chilled (-20 °C) ethanol. After being air-dried, DNA was re-suspended in 60 μ L 1 \times TE buffer and stored at -20 °C prior to use (Section 2.13.4). 1 \times TE buffer was prepared by diluting 10 \times TE buffer (12 g/L TRIS, 4 g/L EDTA, adjusted to pH 8.0 with 1 M HCl and sterilised by autoclaving at 121 °C and 15 psi for 15 min).

2.13.2 Extraction of mitochondrial DNA

Extraction of mitochondrial DNA (mtDNA) was performed based on the methods of Defontaine, *et al.* (1991) and Gibson (1989) with several modifications. Cells pre-grown on YPD media (Section 2.1) were collected by centrifugation at 3,000 rpm for 5 min and the resulting pellet (corresponding to around 0.3 to 0.4 g wet weight) was washed once in sterile RO water and stored at -20 °C for approximately 18 hours prior to analysis. After thawing, cells were washed twice in sterile RO water and re-suspended in 0.5 mL of solution A (1.2 M sorbitol, 50 mM EDTA; Fisher Scientific, UK) containing 10 µL of 2 % β-mercaptoethanol (Sigma, UK) at 37 °C for 10 min. Cells were recovered by centrifugation at 3,000 rpm for 5 min and re-suspended in 1 mL of solution B (0.5 M sorbitol, 10 mM EDTA, 50 mM Tris-HCl, pH 7.5; Fisher Scientific, UK) containing 20 µL of 0.2 mg/mL lyticase (Sigma, UK). The solution was incubated at 37 °C for 1 hour with gentle agitation. Subsequently the suspension was sonicated using an ultrasonic cell disruptor (Soniprep 150, Biosys-Scientific, USA) until the samples became visually clear. The cellular lysate was then centrifuged at 2,000 rpm for 10 min to obtain supernatant, which was isolated into a fresh Eppendorf tube and centrifuged again at 15,000 rpm for 10 min at 4 °C. The resulting pellet was washed three times with solution A and re-suspended in 200 µL buffer comprising 175 µL of solution B, 5 µL of *DNase* (New England Biolabs, UK) and 20 µL of 10× *DNase* buffer (New England Biolabs, UK). The suspension was incubated at room temperature for 10 min before the addition of 1 mL solution A. Subsequently a pellet was obtained by centrifugation at 15,000 rpm for 10 min at 4 °C and re-suspended in 0.5 mL of lysis buffer (100 mM NaCl, 10 mM EDTA, 50 mM Tris-HCl, 1 % Sarkosyl, pH 7.8; Fisher Scientific, UK) containing 5 µL of 2 U/µL *RNase* (New England Biolabs, UK). After incubation for 30 min at room temperature, 0.5 mL of phenol-chloroform (Fisher Scientific, UK) was added and mixed by pipetting. The supernatant was transferred to a fresh Eppendorf tube, to which 0.6 mL of chloroform (Fisher

Scientific, UK) was added. The aqueous phase was obtained by centrifugation at 15,000 rpm for 5 min at 4 °C and transferred to a new Eppendorf tube. 25 µL of 5 M NaCl (Fisher Scientific, UK) and 0.6 mL of isopropanol (Fisher Scientific, UK) were added. The mixture was incubated at room temperature for 30 min and centrifuged for 30 min at 15,000 rpm and 4 °C. The mtDNA pellet was washed once with 0.6 mL of 70 % (v/v) ethanol (Fisher Scientific, UK) and left to air-dry for 15 min. Finally, the pellet was re-suspended in 40 µL molecular grade water (Fisher Scientific, UK) and stored at -20 °C until required (Section 2.13.5).

2.13.3 Quantification of DNA

The quantity and quality of the isolated DNA (Sections 2.13.1-2.13.2) was determined using a ND-1000 spectrophotometer (Nanodrop Technologies Inc., USA), calibrated according to the manufacturer's recommendation. 2 µL of DNA was pipetted onto the pedestal of the instrument and the concentration and purity of DNA was calculated automatically based on the absorbance at 260/280 nm.

2.13.4 Genomic DNA fingerprinting by PCR analysis of inter-delta genomic regions

Genomic DNA fingerprinting was conducted by PCR amplification of yeast inter-delta sequences, using primers delta12/21 (delta12: 5'-TCAACAATGGAATCCCAAC-3' and delta21: 5'-CATCTTAACACCGTATATGA-3'; Eurofins MWG, UK) according to (Legras & Karst, 2003). Reaction components (Table 2.13) were prepared on ice and DNA amplification (Table 2.14) was conducted using a thermal cycler (TC-512, Techne, UK). Amplified DNA fragments were resolved by gel electrophoresis as described in Section 2.13.6.

Table 2.13 PCR components for analysis of inter-delta regions of genomic DNA

<i>Reagent</i>	<i>Per reaction (μL)</i>	<i>20 reaction master mix (μL)</i>	<i>Final concentration</i>
10 μ M Forward primer (delta12)	2.5	50	1 μ M
10 μ M Reverse primer (delta21)	2.5	50	1 μ M
2 \times Phusion Master Mix (New England Biolabs, UK)	12.5	250	1 \times
Molecular grade H ₂ O (Fisher Scientific, UK)	6.5	130	N/A
DNA template (approximately 100 ng/ μ L)	1	N/A	Up to 4 ng/ μ L
Total volume	25	480	N/A

Table 2.14 Cycling condition for analysis of inter-delta regions of genomic DNA

	<i>Temperature</i>	<i>Duration</i>	<i>Number of cycles</i>
<i>Step 1</i>	98 °C	30 sec	1
<i>Step 2</i>	98 °C	10 sec	35
	48 °C	30 sec	
	72 °C	3 min	
<i>Step 3</i>	72 °C	10 min	1
<i>Step 4</i>	4 °C	Hold	N/A

2.13.5 MtDNA fingerprinting by restriction fragment length polymorphism (RFLP)

Restriction endonucleases *XmaI* and *HinfI* (New England Biolabs, UK) were used to digest mtDNA isolated as described previously (Section 2.13.2). Digestion mixtures (Table 2.15) were prepared on ice and samples were incubated at 37 °C for 90 min. Restricted mtDNA fragments were resolved by gel electrophoresis as described in Section 2.13.6.

Table 2.15 Reaction mixture for RFLP analysis (Chemicals supplied by New England Biolabs, UK)

<i>Reagent</i>	<i>Per reaction (μL)</i>
10 U/mL restriction enzyme (<i>XmaI</i> or <i>HinfI</i>)	1
Enzyme buffer	2
1 mg/mL BSA	2
2 U/μL RNase	1
mtDNA template	8
Molecular grade H ₂ O	6
Total volume	20

2.13.6 Agarose gel electrophoresis

DNA fragments obtained by either PCR (Section 2.13.4) or RFLP (Section 2.13.5) were resolved and analysed by agarose gel electrophoresis (Sanger, *et al.*, 1982). A 1.2 % (w/v) agarose gel (Invitrogen, USA) was prepared using 1× TAE buffer (diluted from 50× TAE buffer consisting of 242 g/L TRIS, 57.1 mL/L glacial acetic acid and 18.6 g/L EDTA)

containing 4 μL of 10 mg/mL ethidium bromide (Sigma, UK). Gel electrophoresis was conducted using a standard 20 cm \times 20 cm electrophoresis tank (Anachem, UK) filled with 1 \times TAE buffer.

2.13.6.1 Separation of PCR fragments by gel electrophoresis

10 μL aliquots of PCR product (Section 2.13.4) were mixed with 2 μL of 6 \times loading dye (2.5% Ficoll®-400, 11mM EDTA, 3.3 mM Tris-HCl, 0.017 % SDS, 0.015 % bromophenol blue, pH 8.0; New England Biolabs, UK) and loaded into wells located within the gel. A 7 μL aliquot of DNA marker, 1 Kb or 100 bp ladder (New England Biolabs, UK), was also loaded into a discrete well as a reference. DNA was resolved by electrophoresis at 70 V for an initial 30 min and then 90 V for 1 hour. DNA was visualized under UV light and photographed using a GelDoc® UV transilluminator (UK) and VisionWorksLS software.

2.13.6.2 Separation of mtDNA fragments by gel electrophoresis

In order to resolve mtDNA fragments derived from RFLP digestion (Section 2.13.5), 3 μL of 6 \times loading dye was added into each reaction solution (20 μL) and the entire mixture (23 μL) was pipetted into a well located within the gel. A 1 Kb DNA ladder (New England Biolabs, UK) was used as a molecular weight DNA standard and a voltage of 70 V was applied for 30 min to resolve restriction fragments. MtDNA fragments were visualized under UV light and photographed using a GelDoc® UV transilluminator (UK) and VisionWorksLS software.

2.14 Statistical Analysis

The mean and standard deviation of a data set was calculated using Excel (Microsoft, USA). Statistical analyses were performed using SPSS version 20.0 for windows (Chicago, USA). Data was subjected to one-way analysis of variance (ANOVA) with least significant difference test (LSD) or student's t-test to determine the significant differences between samples. The null hypothesis was that there was no significant difference between data sets,

and differences were considered significant at $P < 0.05$. In this instance, if the P value generated was less than 0.05, the null hypothesis of no significant difference was rejected. Specific statistical tests applied to raw data are indicated in the relevant result sections.

CHAPTER 3: CHARACTERISATION OF BREWING YEAST STRAINS

3.1 Introduction

The central biological agents of beer fermentation are brewing yeasts belonging to the genus *Saccharomyces*. These yeast strains have been selected over many years for their competitive properties including fast growth, efficient sugar utilisation, and their ability to produce ethanol and flavour-active compounds (Piskur, *et al.*, 2006, Lodolo, *et al.*, 2008, Pires, *et al.*, 2014). Brewing yeasts can be divided into two groups referred to as ale and lager strains, named according to their fermented products. Ale brewing strains belong to the species *Saccharomyces cerevisiae* (Pedersen, 1986, Smart, 2007), while lager yeasts are hybrid organisms and are classified as *Saccharomyces pastorianus* (Vaughn-Martini & Martini, 1998, Smart, 2007) (See Section 1.2.1).

The genetic differences between brewing yeast types have resulted in characteristic distinguishing features which can be used to differentiate between lager and ale strains. Lager yeasts exhibit lower permissive growth temperature than ale strains, most likely driven by the presence of genomic DNA derived from a *S. bayanus*-like parental strain (Section 1.2.1), believed to be more cold-tolerant than *S. cerevisiae* (Giudici, *et al.*, 1998, Sato, *et al.*, 2002). Consequently lager yeasts are traditionally used to ferment at temperatures of 8-15 °C and have a maximum growth temperature of between 31.6-34 °C, and an optimum growth temperature of < 30 °C (Giudici, *et al.*, 1998, Boulton & Quain, 2001). Ale yeasts are often used to ferment at comparatively high temperatures (18-25 °C) and have an optimum growth temperature of > 30 °C, with a maximum growth temperature of between 37.5-39.8 °C (Walsh & Martin, 1977, Boulton & Quain, 2001). One of the other main physiological differences between ale and lager yeast is the ability of lager strains to secrete α -galactosidase (also named melibiase) and hence the ability to utilize the sugar melibiose, resulting from the presence of a series of *MEL* genes (Turakainen, *et al.*, 1993). Ale yeast strains do not possess *MEL* genes and consequently are not able to metabolize melibiose (Naumov, *et al.*, 1995).

These phenotypic characteristics enable a simple route to the differentiation of ale and lager strains based on growth mechanics.

Although there are significant differences between ale and lager yeasts, individual strains belonging to each group also display unique characteristics. This is particularly evident for ale yeast which constitutes a diverse range of strains, but is also true of lager yeast (Pedersen, 1986, Timmins, *et al.*, 1998, Smart, 2007). Previous studies have demonstrated that brewing yeasts can vary significantly in terms of their genetic composition, and consequently their phenotypic and physiological characteristics. The effects of these differences are manifested in a diverse number of ways, including variations in growth characteristics, nutritional requirements, the rate at which sugars are assimilated, production of flavour compounds, flocculation properties, and tolerance to a wide range of stress factors. Resistance to stress is particularly important in the brewing process since all fermentations are inherently stressful to some degree. This is particularly true for High Gravity (HG) and Very High Gravity (VHG) fermentations, where yeast cells are subjected to elevated environmental stresses, primarily as a result of increased ethanol concentration and osmotic pressure (Section 1.5). High concentrations of ethanol are accumulated during the latter stage of fermentation and have been reported to have toxic effects on both non-specific and specific sites (Jones & Greenfield, 1987), including the cell membrane (Jimenez & Benitez, 1987, Salgueiro, *et al.*, 1988, Alexandre, *et al.*, 1994, You, *et al.*, 2003) and the mitochondria (Bandas & Zakharov, 1980, Aguilera & Benitez, 1985, Aguilera & Benitez, 1989, Kitagaki, *et al.*, 2007). Osmotic stress has also been suggested to be elevated during HG and VHG brewing, believed to be due to the use of worts with high sugar concentration. Even moderate osmotic stress can result in modifications to cell physiology, metabolism and gene expression (Morris, *et al.*, 1986, Laroche, *et al.*, 2001, Sigler, *et al.*, 2009, Verbelen, *et al.*, 2009) and, when stress is severe or damage has accumulated to intolerable levels, this can lead to a deterioration in

yeast activity and fermentation performance (Beney & Gervais, 2001, Jenkins, *et al.*, 2003, Verbelen, *et al.*, 2009).

In this study, we assess a number of production yeast strains for their genetic and phenotypic characteristics, including analysis of permissive growth temperature and ability to secrete melibiase. Molecular methods for the differentiation of strains were applied based on analysis of mitochondrial DNA (mtDNA). In addition, each strain was analysed for physiological characteristics related to HG and VHG fermentations. This was performed primarily to identify differences between organisms and their suitability for fermentation, a measure which can also often form part of the quality assurance verification of culture yeasts (Lodolo, *et al.*, 2008), but also to benchmark each strain for further studies. Therefore, the effects of ethanol and osmotic stress on yeast growth were investigated in order to obtain an overview of the phenotypic characteristics of the brewing lager and ale yeast strains selected.

3.2 Results

Two lager yeast strains, designated Lager1 and W34/70, and two ale strains, designated NCYC1332 and M2 were used in this study (Section 2.1). Yeasts were analysed for specific phenotypic and genetic characteristics with the aim of identifying each strain to the species and strain level, as well providing insight into their physiological characteristics. Differentiation of brewing yeast to the species level was determined by investigating permissive growth temperature (Section 3.2.1.1) and the ability to secrete melibiase (also named α -galactosidase), the enzyme responsible for cleaving melibiose (Section 3.2.1.2). Strain differentiation was performed based on restriction endonuclease fingerprinting of mtDNA (Section 3.2.1.3). In addition, with respect to yeast stress tolerance, the effect of ethanol stress on yeast growth was determined using spot plate analysis (Section 3.2.2) and

the impact of osmotic stress on growth was determined using both spot plate (Section 3.2.3.1) and kinetic growth analyses (Section 3.2.3.2).

3.2.1 Determination of brewing yeast classification

3.2.1.1 Permissive growth temperature of brewing yeast strains

Analysis of growth temperature (Section 2.3.1) is a traditional means of differentiating lager and ale strains, since it is known that *S. cerevisiae* and *S. pastorianus* have unique permissive growth temperatures. To establish the maximum growth temperature of two putative lager strains (Lager1 and W34/70) and two putative ale strains (NCYC1332 and M2), cells were pre-grown in liquid YPD media, streaked onto agar plates and incubated at 25 °C, 34 °C, and 37 °C. The extent of growth was recorded by colony formation (Figure 3.1) and used to confirm the species and brewing classification of each strain.

After 5 days incubation, strains NCYC1332 and M2 were observed to produce colonies at 25 °C, 34 °C, and 37 °C, whilst strains Lager1 and W34/70 were culturable only at temperatures lower than 37 °C. Interestingly, it was observed that the latter two strains exhibited relatively different growth patterns at 34 °C, with Lager1 appearing to display a comparatively reduced ability to form colonies (Figure 3.1C). However both strains were completely inhibited at 37 °C (Figure 3.1D).

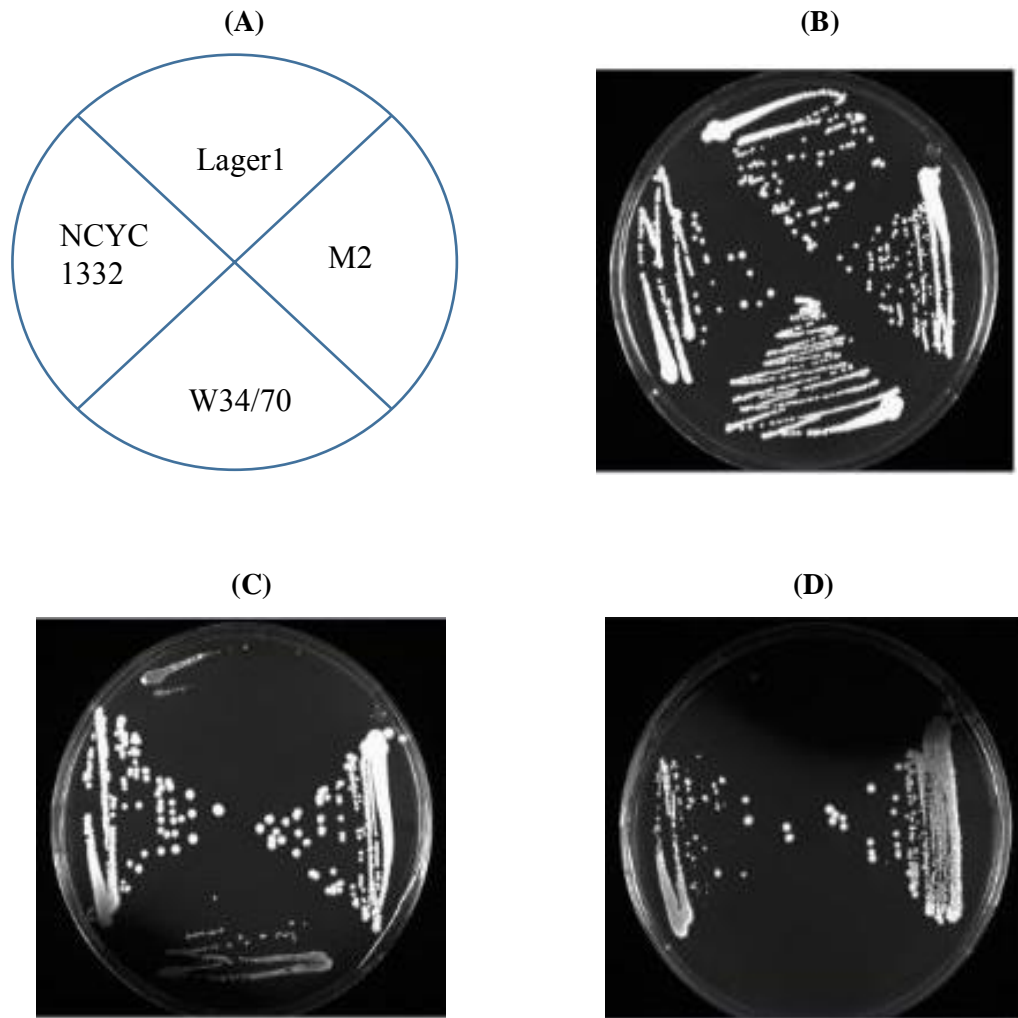


Figure 3.1 Growth of yeast strains on YPD agar at different temperatures. (A) layout of the strains (B) colony growth at 25 °C, (C) colony growth at 34 °C, (D) colony growth at 37 °C.

3.2.1.2 Ability of yeast to utilise melibiose

The X- α -gal test (Box, *et al.*, 2012) can be used to determine the capacity of yeast to cleave the melibiose homolog X- α -gal (5-bromo-4-chloro-3-indolyl- α -D-galactopyranoside), resulting in the development of a blue coloration derived from indol (Box, *et al.*, 2012). This reaction functions as a means of identifying lager yeasts based on their ability to secrete α -galactosidase (melibiase) and cleave X- α -gal as described in Section 2.3.2.

After 30 min incubation, a colour change from cream-white to blue was observed to occur for strains Lager1 and W34/70, whilst there was no colour change for NCYC1332 or M2 (Figure 3.2). In order to confirm results, each strain was incubated for an extended period of time; however identical results were observed when samples were re-examined after 24 hours and 7 days incubation (data not shown).

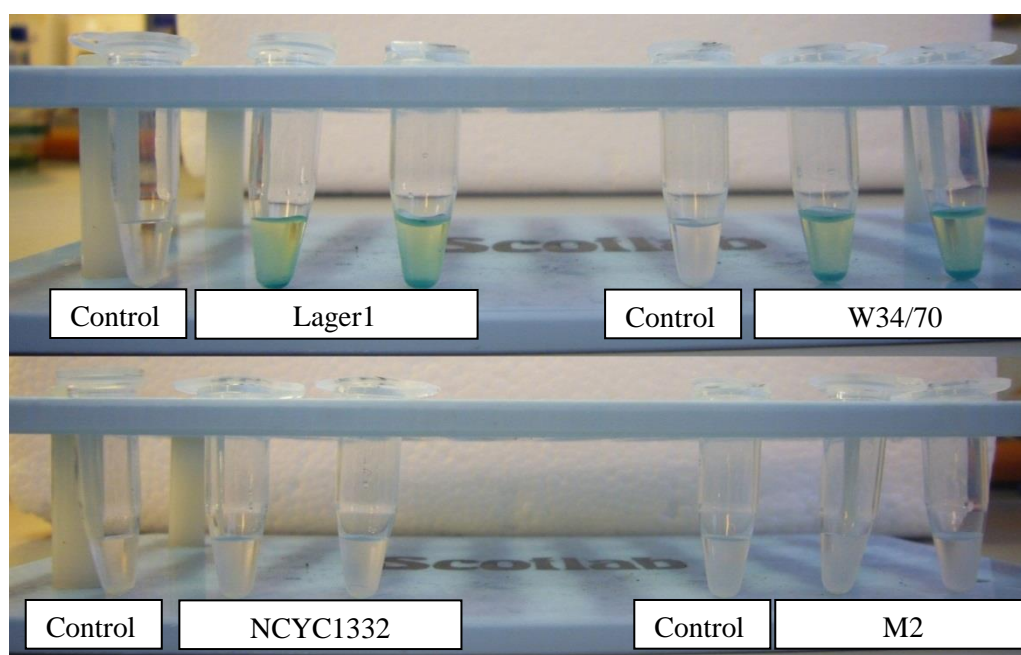


Figure 3.2 Analysis of the ability of yeast strains to utilize melibiose. Formation of blue colour indicates the presence of α -galactosidase (melibiase), responsible for cleaving melibiose into its constituent parts. Yeast suspensions incubated without X- α -gal were included as negative control samples.

To confirm the data obtained from X- α -gal analysis, each brewing strain was also cultivated on liquid media containing either melibiose or glucose (control) as a sole carbon source. Utilization of each carbon source was indicated by the production of gas collected in Durham tubes and a colour change from blue to yellow, corresponding to CO₂ and acid production

(indicated by the conversion of the pH indicator bromothymol blue), respectively (Section 2.3.3). As expected, utilization of glucose was demonstrated by each of the four strains (Table 3.1), however only Lager1 and W34/70 were able to metabolise when melibiose was present as a sole source of carbon, confirming their classification as lager yeast (Table 3.1).

Table 3.1 Yeast ability to utilize melibiose (+, positive; -, negative)

<i>Carbon source</i>	<i>Melibiose</i>		<i>Glucose</i> (<i>Positive control</i>)		<i>Water</i> (<i>Negative control</i>)	
	Gas	Colour	Gas	Colour	Gas	Colour
Lager1	+	+	+	+	-	-
W34/70	+	+	+	+	-	-
NCYC1332	-	-	+	+	-	-
M2	-	-	+	+	-	-

3.2.1.3 Differentiation of yeast strains using mtDNA RFLP

Although analysis of genomic DNA has been demonstrated to be a useful tool for yeast identification, previous studies have indicated that the evolution of yeast mtDNA occurs at a relatively rapid rate when compared to nuclear DNA (Brown, 1981). Consequently, it has been argued that analysis of mtDNA has the potential to be more sensitive for yeast strain differentiation than total genomic DNA (Lee, *et al.*, 1985). Indeed, digestion of mtDNA with restriction enzymes including *HinfI* has been proved to be an effective tool in identification of commercial wine yeast strains (Querol, *et al.*, 1992, Fernandez-Espinar, *et al.*, 2001, Schuller, *et al.*, 2004). However, despite significant work in other industries, RFLP analysis of mtDNA in brewing strains using enzymes *HinfI* and *XmaI* has not been reported. In order to further

characterise strains used in this study, each yeast was investigated by RFLP analysis of mtDNA using *XmaI* and *HinfI* (Section 2.13.5) to produce an mtDNA fingerprint of each strain.

Yeast strains Lager1 and W34/70 displayed similar banding patterns when digested using both *XmaI* and *HinfI*. With *XmaI*-digestion, clear bands were observed at approximately 1.3, 1.8, 2.5, 4.0, 6.0, 8.0, 10.0 Kb for both strains (Figure 3.3). With *HinfI*-digestion, a number of clear bands were observed, particularly evident at 3.0 and 5.0 Kb, with a number of additional bands between 1.0 and 3.0 Kb in size (Figure 3.4). In comparison, the yeast strains NCYC1332 and M2 showed a single DNA band of approximately 14.0 Kb with *XmaI*-digestion (Figure 3.3). However, both of these yeasts displayed a unique mtDNA RFLP profile when digested using *HinfI*: a number of discriminatory bands were observed most obviously at approximately 1.0, 3.0 and 5.0 Kb (Figure 3.4). While the two ale strains examined were therefore readily differentiated, the RFLP profiles obtained from analysis of lager strains were similar and did not allow for strain differentiation. It is suggested that this may be due to the close genetic heritage of lager strains and indicates that both strains were particularly similar in terms of their mtDNA composition and structure.

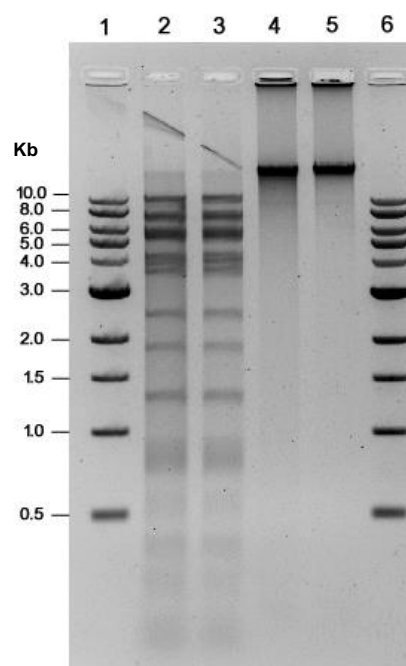


Figure 3.3 MtDNA RFLP analysis of brewing yeast strains using restriction enzyme *XmaI*. Lane 1, 6: molecular weight marker; lane 2: Lager1; lane 3: W34/70; lane 4: NCYC 1332; lane 5: M2.

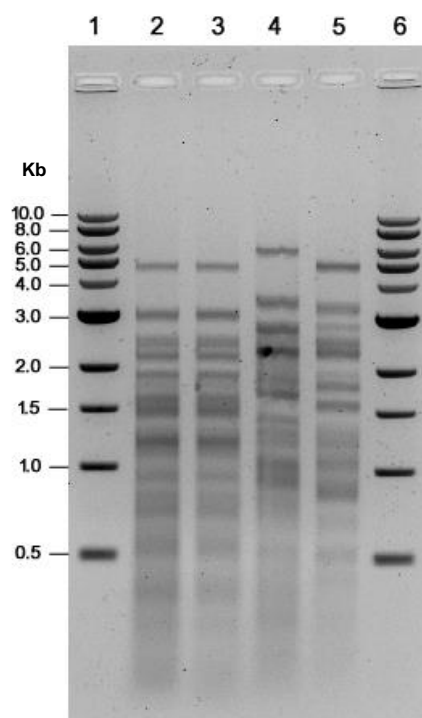


Figure 3.4 MtDNA RFLP analysis of brewing yeast strains using restriction enzyme *HinfI*. Lane 1, 6: molecular weight marker; lane 2: Lager1; lane 3: W34/70; lane 4: NCYC 1332; lane 5: M2.

3.2.2 Ethanol tolerance of brewing yeast strains

In order to assess the effect of ethanol on yeast growth, a spot plate assay was used (Section 2.5.1). This method allows strains to be compared based on their ability to generate colonies on nutrient agar plates under specific conditions. In this study, serially diluted (1:10, starting $OD_{600} = 1.0$) cell suspensions (3 μ L) were spotted onto wort agar plates supplemented with 0-20 % (v/v) ethanol and incubated at 25 °C. The extent of growth was recorded after incubation for 48, 72, 96 and 120 hours (Figure 3.5).

After 48 hours incubation, the amount of growth observed for each of the four strains was similar, irrespective of the growth conditions applied. A gradual inhibition effect was observed when increasing the concentration of ethanol from 0 to 10 % (v/v). It was also noticed that at 15 % (v/v) ethanol, colonies were only produced when cultures were inoculated at high concentrations, and not when these were diluted. Furthermore, at 20 % (v/v) ethanol, colonies were difficult to detect visually, irrespective of cell concentration. After both 72 and 96 hours of incubation, a similar pattern of results was obtained (Figure 3.5). The greatest amount of growth was observed when strains were incubated at ethanol concentrations of between 0 and 10 % (v/v). There was only limited growth at 15 % (v/v) ethanol and no sign of growth at 20 % (v/v) ethanol. After 120 hours of incubation, identical results were observed when strains were cultivated on 0-10 % (v/v) ethanol (Figure 3.5). However, it was interesting to observe that on 15 % (v/v) ethanol the strain Lager1 produced the greatest amount of growth, whilst W34/70 and NCYC1332 were similar, albeit at a lower level. In contrast, the yeast strain M2 showed the least amount of growth of any of the four strains when cultivated on 15 % (v/v) ethanol. Finally, growth on 20 % (v/v) ethanol continued to be poor, indicating that strains were unable to survive, or struggled to produce biomass at this ethanol concentration.

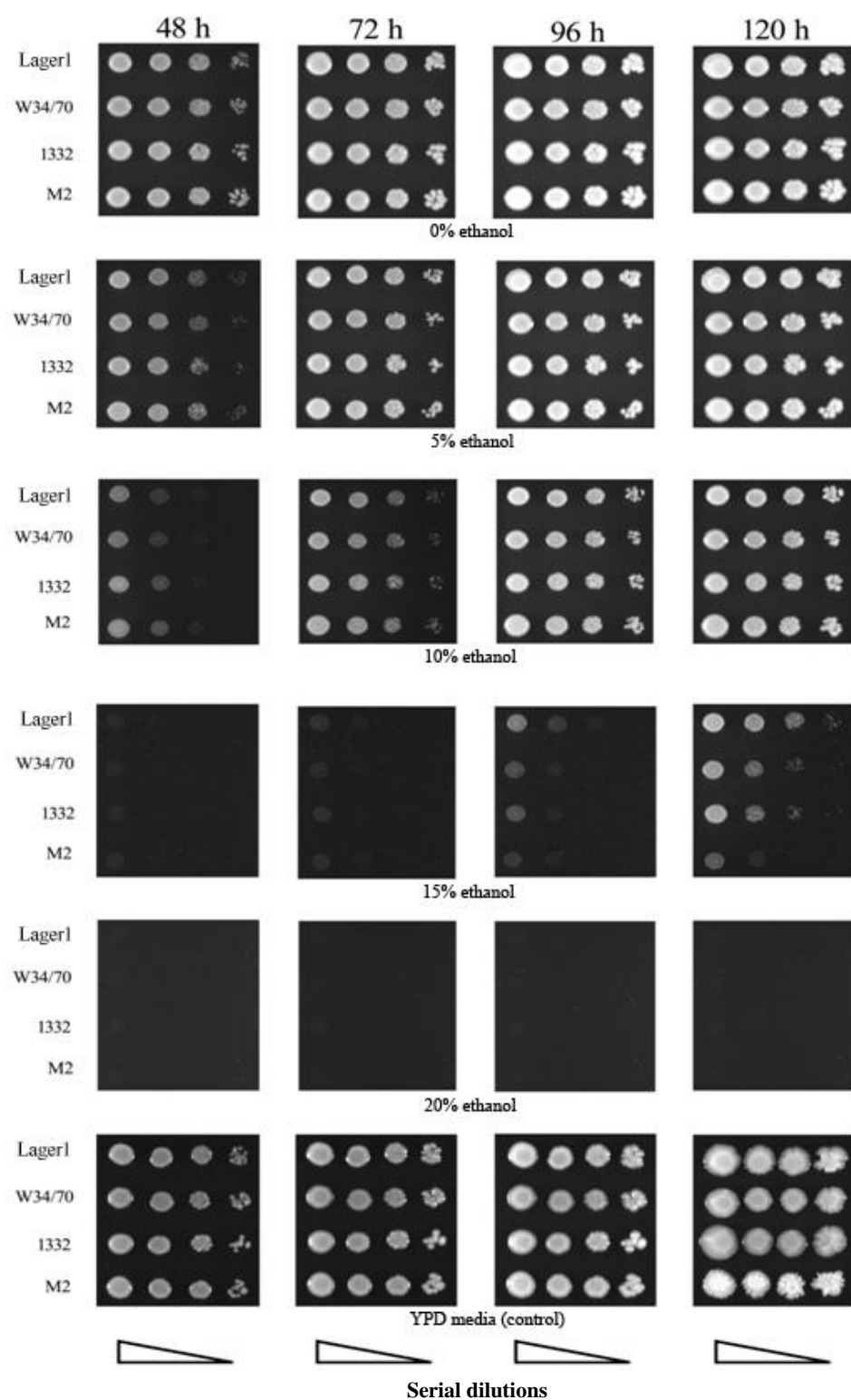


Figure 3.5 Growth of yeast strains on different ethanol concentrations at 25 °C. Sequential dilutions (1:10, starting OD₆₀₀ 1.0) were spotted onto wort-ethanol plates (13 °P brewer's wort supplemented with 0-20 % [v/v] ethanol) at a volume of 3 µL. The extent of growth was recorded after incubation for 48, 72, 96 and 120 hours. YPD media was used as a positive control.

3.2.3 Osmotic tolerance of brewing yeast strains

3.2.3.1 Effect of osmotic stress on yeast growth on solid media

In order to obtain an overview of the effect of osmotic stress on cell growth, a spot plate assay was used to investigate the ability of each strain to generate colonies on nutrient agar plates under defined osmotic stress conditions (Section 2.5.1). This was conducted on solid medium: 50 % (v/v) 13 °P brewer's wort (final gravity 7.5 °P) supplemented with 50 % (v/v) sorbitol solution to achieve a series of final sorbitol concentrations (0, 6, 12, 18, 24 and 30 % , w/v). Sorbitol was chosen as an osmotic effector since it is not directly toxic to yeast and is not assimilated by the yeasts studied. The main effect of sorbitol here was to provide a series of increasing osmotic challenges, a technique which has previously been employed to examine osmo-sensitivity in a range of yeast strains (Pratt, *et al.*, 2003, Wojda, *et al.*, 2003). 3 µL of serially diluted (1:10, starting OD₆₀₀ = 1.0) cell suspensions were spotted onto the wort-sorbitol plates and incubated at 25 °C. The formation of colonies on plates was used for comparison between yeast strains at different wort-sorbitol concentrations (Figure 3.6).

After 48 hours incubation, the amount of growth was similar for the four yeast strains, irrespective of the osmotic conditions applied. Agar plates containing 0-18 % (w/v) sorbitol were able to support growth, however reduced colony formation was observed at higher dilutions (lower inoculum). When sorbitol concentration was elevated further, to 24 and 30 % (w/v), colonies were only produced when cells were taken from the first three dilutions and not when higher dilutions were applied. After 72, 96 and 120 hours incubation an identical pattern of growth was observed, albeit with higher levels of growth for each strain, further demonstrating that yeast growth was negatively affected by increasing osmotic shock induced by sorbitol.

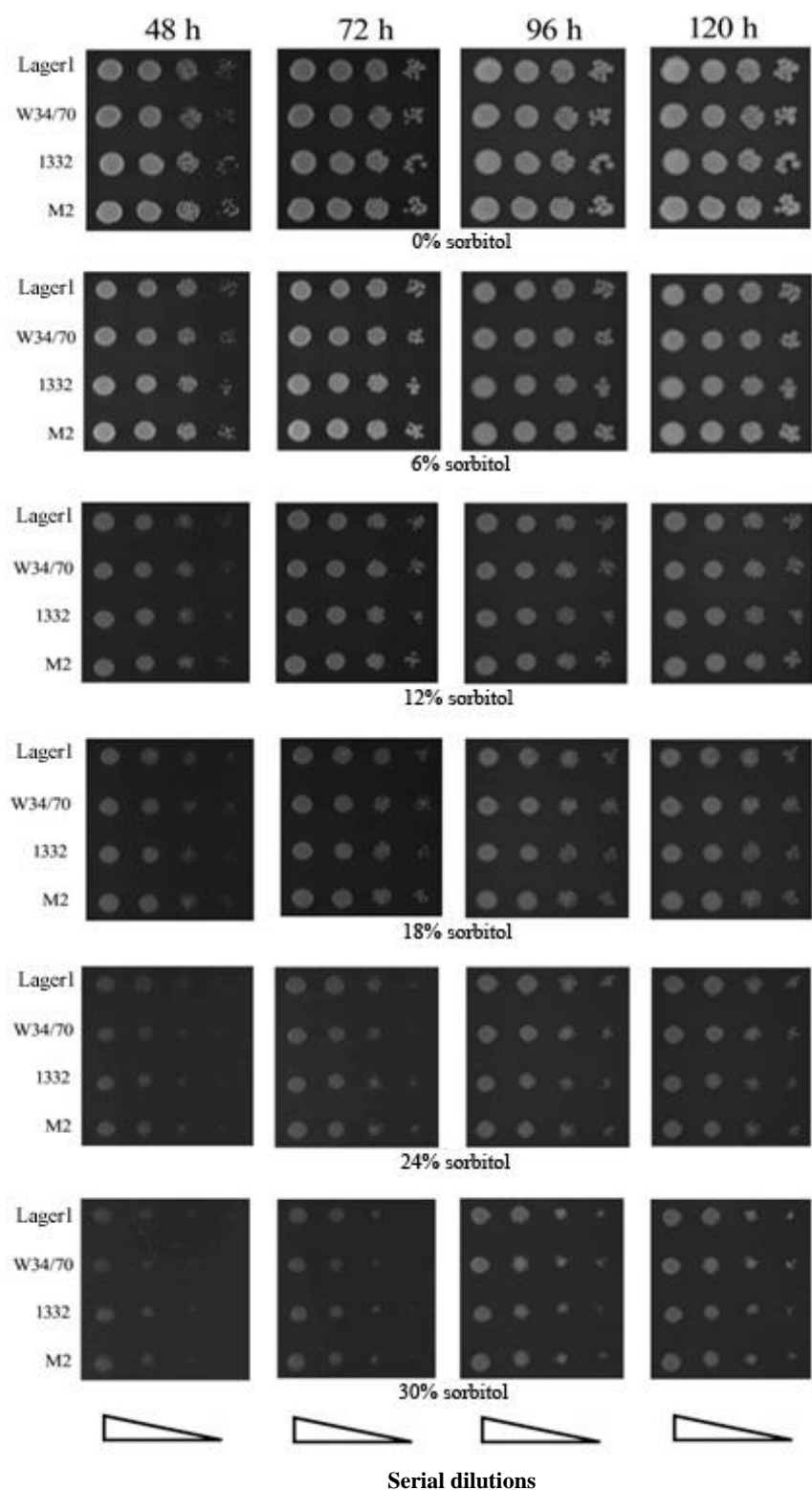


Figure 3.6 Growth of yeast strains on different wort-sorbitol concentrations at 25 °C. Sequential dilutions (1:10, starting OD₆₀₀ 1.0) were spotted onto wort-sorbitol plates (50 % [v/v] 13 °P brewer's wort supplemented with 50 % [v/v] sorbitol to achieve final sorbitol concentration of 0-30 % [w/v]). The extent of growth was recorded after incubation for 48, 72, 96 and 120 hours.

3.2.3.2 Effect of osmotic stress on yeast growth on liquid media

Although spot plate analysis is a useful tool for determining the absolute limits of growth under specified conditions, it only provides limited information on the response of cells within a population. In order to achieve a more accurate indication of the effect of osmotic stress on cell growth dynamics, including lag phase and time to reach stationary phase, each yeast strain was cultivated in liquid media containing 50 % (v/v) 13 °P brewer's wort (final gravity 7.5 °P) supplemented with sorbitol to achieve final sorbitol concentrations of 0-30 % (w/v). Kinetic growth was determined by absorbance at 600 nm using a 96-well plate reader (Section 2.5.2).

In general, an extended lag phase and concomitantly longer time to reach stationary phase was observed for each of the four yeast strains during growth on increasing sorbitol concentrations (Figures 3.7-3.8). When cells were incubated in media containing 0-12 % (w/v) sorbitol, the lag phase occurred between approximately 0-12 hours and the time required to reach stationary phase was about 30 hours, whereas in media containing 18 and 24 % (w/v) sorbitol, the lag phase was observed to broadly extend from 0-19 hours and 0-24 hours respectively. Similarly, the time required to reach stationary phase was extended from 30 hours (0-12 % sorbitol) to 40 hours (18 % sorbitol) and 55 hours (24 % sorbitol). Cells cultivated in 30 % (w/v) sorbitol exhibited the longest lag phase (approximately 30 hours), as well as the slowest growth overall (Figures 3.7-3.8).

The four yeast strains examined also showed a decrease in the maximum amount of growth when subjected to increasing sorbitol concentrations. Lager strains were observed to be affected even by a low concentration of sorbitol, for example, at 6 % (w/v) sorbitol the total growth (OD_{600}) was observed to decrease from 0.8-0.9 to 0.4-0.5 (Figure 3.7). However, the same phenomenon was not detected with ale strains where a sorbitol concentration of 18 %

(w/v) was required before the same kind of effect on population growth was seen (Figure 3.8). Despite this, it is also interesting to note that ale strains exhibited a gradual decrease in population limit from 0 % to 24 % (w/v) sorbitol conditions, an effect which was most pronounced for strain NCYC1332 (Figure 3.8A). In contrast, although lager strains showed lower growth than NCYC1332 at 0 % (w/v) sorbitol culture, their maximum growth was similar when the cells were incubated in 6-24 % (w/v) sorbitol conditions, particularly for strain W34/70 (Figure 3.7B). Moreover, the most serious effect of osmotic stress on growth was observed at 30 % (w/v) sorbitol, characterised by restricted growth and minimum biomass production for all strains.

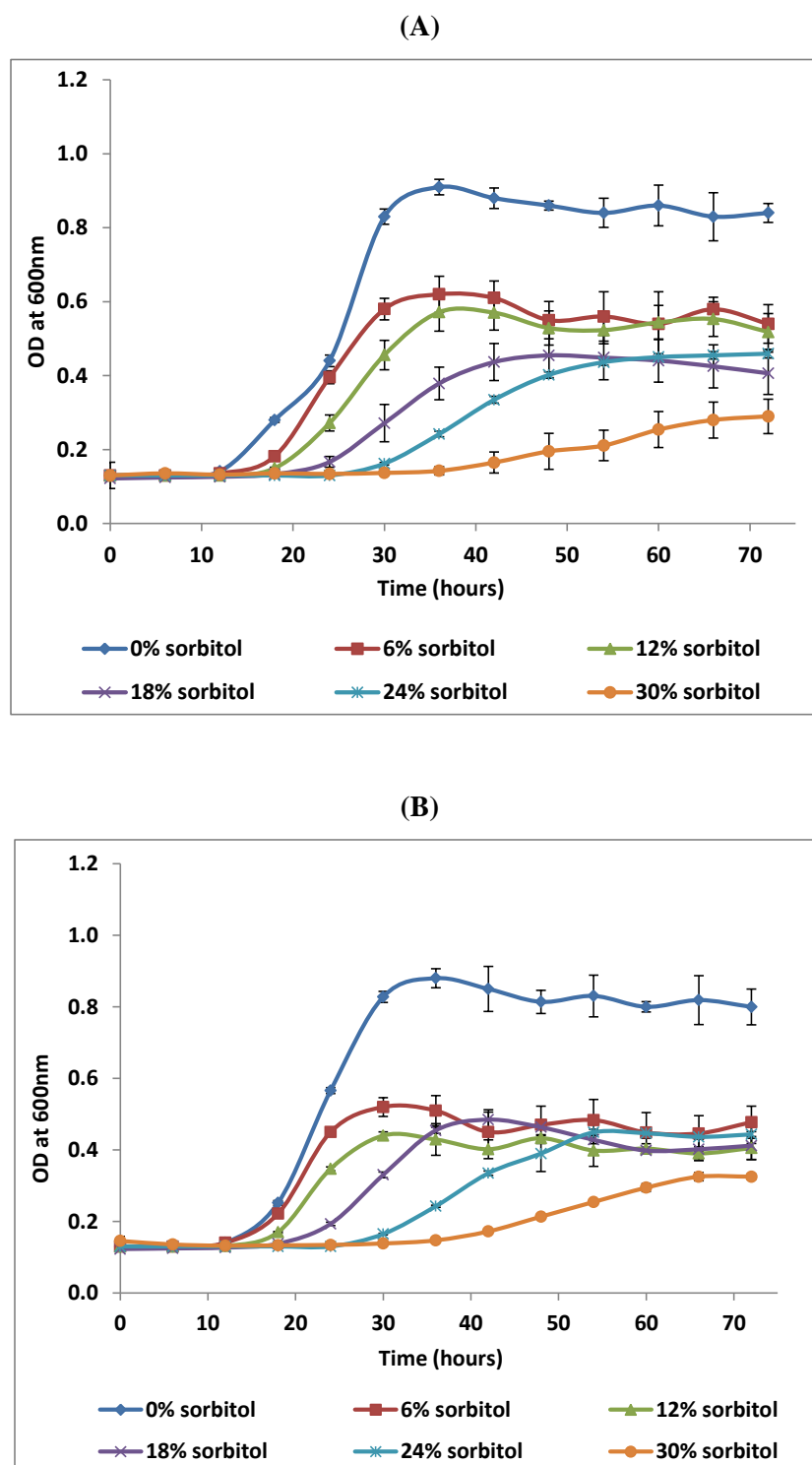


Figure 3.7 Growth curve of lager strains Lager1 (A) and W34/70 (B) under different wort-sorbitol concentrations. 3 μ L of cell suspensions ($OD_{600} = 1.0$) were inoculated into 95 μ L wort-sorbitol media (50 % [v/v] 13 °P brewer's wort supplemented with 50 % [v/v] sorbitol to achieve final sorbitol concentration of 0-30 % [w/v]). Cell growth was determined using a 96-well plate reader by measurement of absorbance at 600 nm every 6 hours after suspending the cells. Data represents the mean of triplicate samples, with error bars showing the standard deviation.

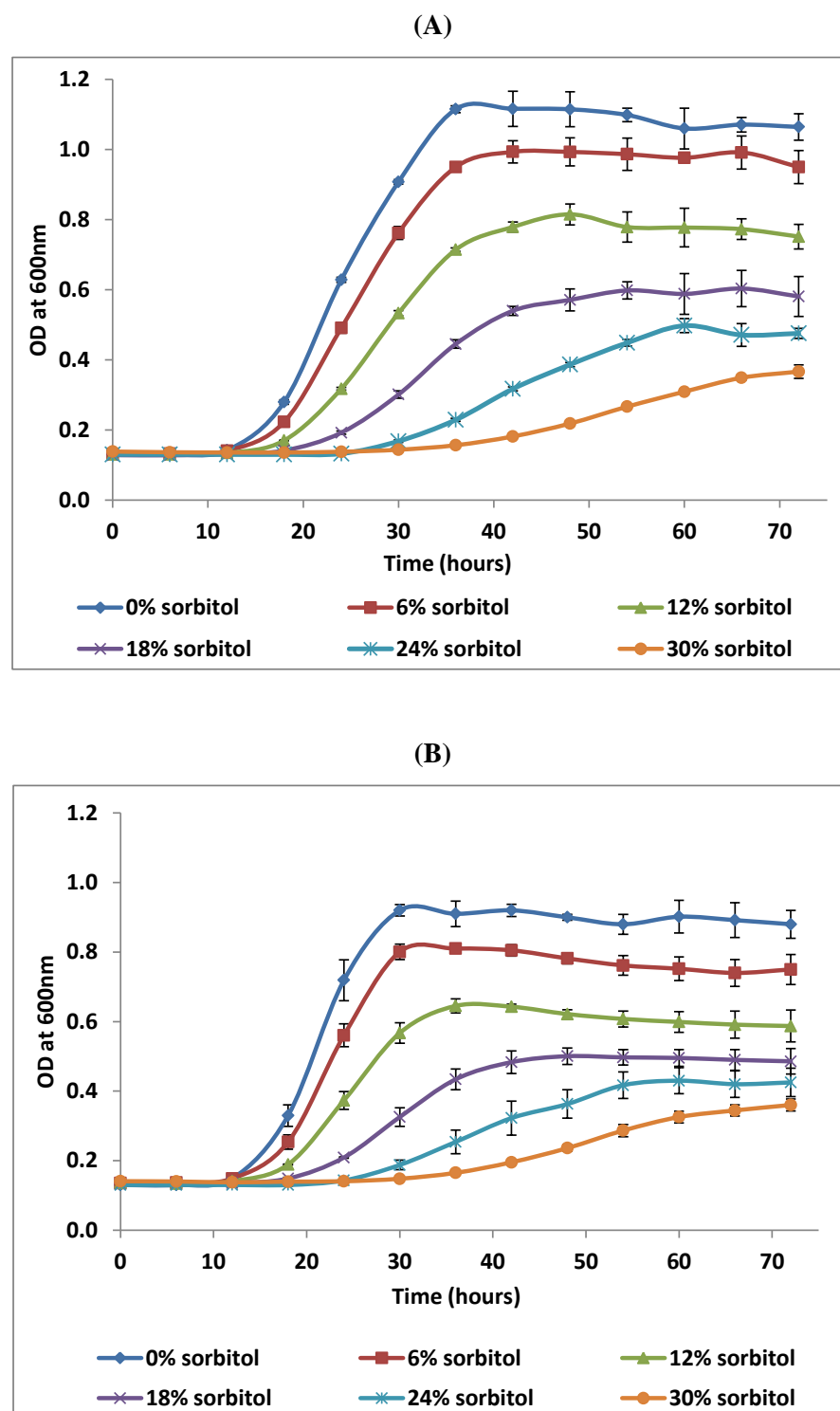


Figure 3.8 Growth curve of ale strains NCYC1332 (A) and M2 (B) under different wort-sorbitol concentrations. 3 μ L of cell suspension ($OD_{600} = 1.0$) were inoculated into 95 μ L wort-sorbitol media (50 % [v/v] 13 °P brewer's wort supplemented with 50 % [v/v] sorbitol to achieve final sorbitol concentration of 0-30 % [w/v]). Cell growth was determined using a 96-well plate reader by measurement of absorbance at 600 nm every 6 hours after suspending the cells. Data represents the mean of triplicate samples, with error bars showing the standard deviation.

3.3 Discussion

Brewery yeast is the most important component in beer production, contributing to the production of ethanol, aromas and flavours, as well as having a significant effect on other organoleptic properties. The characterization of yeast strains is important to gain a general understanding of the strains under investigation, and also provides insight into the basic physiology of yeast prior to further analysis in this thesis.

Although analysis of permissive growth temperature and melibiase secretion does not provide unequivocal identification of brewing strains, these methods are effective approaches in typing ale and lager yeast. Indeed, the results of the present study showed that analysis of growth at 37 °C and ability to cleave X- α -gal test were both rapid and sensitive methods for differentiating between lager and ale yeast strains. Consequently, by employing these methods it was possible to confirm the brewing yeast classification of each of the four strains used in this study as either ale or lager. In an attempt to identify isolates to the strain level, RFLP analysis of mtDNA was applied using *XmaI* and *HinfI* probes. The two ale strains were differentiated (showing unique mtDNA fingerprint) based on this technique with the *HinfI* probe, but not with *XmaI*. The latter resulted in un-cut DNA bands, probably due to the lack of specific restriction site in the isolated mtDNA. In contrast, the two lager strains were not able to be differentiated (showing the same mtDNA fingerprint) using mtDNA RFLP analysis at all, indicating that they may be particularly closely related. Despite this, the two lager strains did appear differ in terms of their response to stress factors, suggesting that they do represent two individual strains. Additional restriction enzymes may be required to distinguish these strains, or alternatively other approaches could be taken including analysis of *TyI* element (Pedersen, 1994, Schofield, *et al.*, 1995, Wightman, *et al.*, 1996), *DEXI* gene (Schofield, *et al.*, 1995), D1/D2 region of 26S rDNA (Huang, *et al.*, 2010) or the karyotypes (Jenkins, *et al.*, 2010) within the yeast genomic DNA.

Characterisation of strains for ethanol and osmotic tolerance indicated that the ale and lager yeasts used in this study were able to withstand 0-10 % (v/v) ethanol. However, exposure of yeast to very high ethanol concentrations (15 % and 20 %, v/v) resulted in severe inhibition of growth. This observation supports previous reports which have shown that exposure of cells to ethanol stress (16 %, v/v) induces the global stress response in yeast (Majara, *et al.*, 1996a, Majara, *et al.*, 1996b, Mahmud, *et al.*, 2010). With respect to osmotic tolerance, analysis of yeast growth on supplemented agar (spot plates) was performed to determine the ability of yeast to divide under a range of osmotic pressures. As expected, the increasing sorbitol-induced osmotic pressure was shown to have a negative effect on yeast growth, an effect which was particularly apparent when sorbitol concentrations above 18 % (w/v) were applied. At 24 % (w/v) sorbitol yeast strains grew slowly; in excess of 72 hours was required for visible colonies to be formed irrespective of the concentration of the starting inoculum. A similar pattern was observed at 30 % (w/v) sorbitol, where growth was retarded for each of the strains examined. However, despite the negative impact of osmotic stress, the results also indicated that cells within the population were not killed directly, but were able to protect themselves against the conditions applied, as evidenced by eventual growth even at high sorbitol concentrations. Examination of growth curves in liquid media yielded comparable results. For the ale and lager strains examined, increasing sorbitol concentrations resulted in an extended lag phase and a concomitantly longer time to reach stationary phase, as well as reduced maximum growth. It was noted that lager strains were more affected at lower sorbitol concentrations than ale strains, suggesting that the lager strains investigated here may be more sensitive to osmotic pressure than the ale yeasts. However, despite this observation, all yeast strains showed a similar response to hyper-osmotic conditions (30 % [w/v] sorbitol) with similar levels of growth on both solid and liquid media.

3.4 Conclusion

In this chapter, two lager-type (Lager1 and W34/70) and two ale-type (NCYC1332 and M2) brewing yeast strains were characterized based on differences in permissive growth temperature and the presence of melibiose (α -galactosidase), as well as mtDNA RFLP profiles. Furthermore, each strain was assessed for their capacity to tolerate stress factors associated with HG and VHG brewing: ethanol and osmotic stress. From this study it was clear that increasing ethanol levels had a gradual inhibition effect on yeast growth. The data presented here indicates that yeast strains are able to withstand ethanol within the range of 0-10 % (v/v); however, outside of this range ethanol was observed to significantly inhibit cell growth and may ultimately lead to cell death. Under wort-sorbitol induced osmotic pressure, increasing sorbitol concentration (0-30 %, w/v) was shown to have a negative effect on yeast growth, which was particularly apparent for colony formation when sorbitol concentrations above 18 % (w/v) were reached. Performing a similar set of experiment using liquid media showed that an extended lag phase and reduced maximum growth were characteristic effects of increasing the degree of osmotic pressure. In addition, lager strains were more affected by lower levels of osmotic stress than the ale strains studied. It is suggested that these differences in osmotic tolerance may be a result of unique cellular responses in each strain, which will be investigated in more detail in Chapter 6. Furthermore, the characterisation of each brewing yeast strain provides preliminary information to support further investigation into yeast physiology and fermentation performance during standard, HG and VHG fermentations (Chapters 4-5).

**CHAPTER 4: HIGH GRAVITY AND VERY HIGH
GRAVITY BREWING FERMENTATIONS: KEY
PERFORMANCE INDICATORS**

4.1 Introduction

High Gravity (HG) brewing is a well-established technique where worts of high sugar concentration (15-20 °P) are employed to produce high alcohol beer, which is then adjusted to sales-gravity by dilution (Stewart, 2010). This method is increasingly attractive as an effective strategy towards enhanced process productivity, reduced investments and overall energy cost savings within the brewing industry (Stewart, 2009, Stewart, 2010, Puligundla, *et al.*, 2011). Due to the success of this approach, there are efforts to explore the possibility of using Very High Gravity (VHG) worts of 20-25 °P (Vidgren, *et al.*, 2009, Gibson, 2011). However, in order to achieve this there are a number of problems which must be overcome, related to the performance of the yeast culture and the characteristics of the final product. Some of the reported effects of HG and VHG brewing include decreased foam stability (Cooper, 1998, Brey, 2004), poor hop utilization (Stewart, 2010), longer fermentation times (Boulton & Quain, 2001), as well as inconsistencies in final product flavour matching (Stewart, 2009, Stewart, 2010, Puligundla, *et al.*, 2011). Furthermore, the use of highly concentrated worts and the transition to a high ethanol environment is concomitant with a number of biological stress factors, which could have a profound influence on yeast quality during fermentation leading to a reduction in the number of serial repitching (Stewart, 2009, Stewart, 2010, Puligundla, *et al.*, 2011, Dekoninck, *et al.*, 2012, Zhuang, *et al.*, 2012, Zhuang, *et al.*, 2013).

There have been several previous studies on the relationship between wort gravity and fermentation performance (Younis & Stewart, 1999, Saerens, *et al.*, 2008, Sigler, *et al.*, 2009, Dekoninck, *et al.*, 2012, Lei, *et al.*, 2012, Yu, *et al.*, 2012). The primary focus of these reports has centred mainly on the relationship between the use of HG and VHG worts and flavour biosynthesis, including the effects of wort carbohydrate profiles on volatile production (Younis & Stewart, 1999), the impact of wort gravity and nitrogen content on flavour balance

(Lei, *et al.*, 2012), and the effect of gene expression on flavour biosynthesis (Saerens, *et al.*, 2008). Comparisons of varying wort gravity on fermentation rate, yeast vitality and sedimentation, beer haze and clarification (Pidcocke, *et al.*, 2009, Sigler, *et al.*, 2009), as well as energy charge and glycolytic enzyme activity (Yu, *et al.*, 2012) have also been investigated. These reports have ultimately suggested that increasing wort density may greatly affect yeast carbon metabolism. This is particularly important since carbohydrates are the most abundant wort nutrient and the majority of fermentation products are carbon-based including ethanol, carbon dioxide, glycerol, and to a lesser extent other metabolites (Section 1.4.5). In order to eliminate or reduce detrimental effects associated with extreme environmental conditions, brewing yeasts respond to their environment by shunting carbon into different metabolic end products (Gibson, *et al.*, 2007), which can assist in the protection of cells but may also impact on final ethanol yield. However, the precise influence of HG and VHG brewing on carbohydrate utilisation and carbon accumulation has not been investigated.

In this study, the effect of wort gravity and pitching rate on key performance indicators was investigated. In particular, the effect of HG and VHG brewing on fermentation performance, carbon end products and yeast quality will be explored using a series of lab-scale fermentations. It is anticipated that the data presented here will provide a greater understanding of the response of yeast to HG and VHG conditions, potentially leading to process optimisation in the future.

4.2 Results

Two lager (Lager1 and W34/70) and two ale (NCYC1332 and M2) brewing yeast strains were examined in this study (Section 2.1). Fermentations were conducted as described in Materials and Methods (Section 2.6). Commercial 25 °P wort was obtained from Molson Coors Brewing Company (Burton-on-Trent, UK) and was diluted to achieve 18 °P (HG) and

24 °P (VHG) worts. Commercial 13 °P wort was also provided and used as a standard gravity control. Each of the worts was analysed for sugar profile and free amino nitrogen level as detailed in Table 4.1. In order to mimic industrial fermentation conditions as closely as possible, 18 °P and 24 °P worts were oxygenated whilst 13 °P wort was aerated for a couple of hours prior to pitching as described in Section 2.6.2.

Table 4.1 Sugar composition and free amino nitrogen content of worts used in this study

<i>Wort gravity (°P)</i>	<i>Fructose (g/L)</i>	<i>Glucose (g/L)</i>	<i>Sucrose (g/L)</i>	<i>Maltose (g/L)</i>	<i>Maltotriose (g/L)</i>	<i>FAN (mg/L)</i>
13	2.7 ± 0.5	10.2 ± 0.2	2.1 ± 0.5	57.9 ± 2.5	19.8 ± 2.6	170 ± 15
18	3.7 ± 0.6	13.2 ± 1.8	3.0 ± 0.8	80.9 ± 3.8	25.8 ± 2.5	195 ± 13
24	5.7 ± 0.9	20.2 ± 0.5	5.6 ± 1.7	107.4 ± 4.1	35.9 ± 3.1	260 ± 21

Yeast slurries were propagated in YPD media (Section 2.6.4) and used to pitch wort based on five different experimental conditions as described in Table 4.2. Firstly, fermentations were pitched at 1.5×10^7 viable cells/mL, irrespective of initial wort gravity ('standard' pitching rate). In addition, a series of 18 °P and 24 °P fermentations were also initiated by pitching 1.0×10^6 viable cells/mL per degree Plato, an adjusted pitching rate based on industrial 'rule of thumb' (Stewart, 2009). The experimental programme was conducted in sequence. Fermentations were conducted using lab-scale miniature fermenters (100 mL wort volume) with constant stirring (350 rpm); the homogeneous nature of the fermenters enabled simultaneous fermentations under identical conditions to be conducted. Fermentations were conducted for 120 hours and assessed in triplicate by destructive sampling at approximately 0, 3, 15, 24, 48, 65, 90 and 120 hours for the respective analyses (Sections 4.2.1-4.2.2).

Table 4.2 Fermentation conditions. Abbreviations listed under test conditions are used throughout the text to refer to the appropriate set of experiments.

<i>Test conditions</i>	<i>Wort gravity (°P)</i>	<i>Oxygen/aeration conditions</i>	<i>Pitching rate (Viable cells per mL)</i>
13P15M	13	Aeration for 24 hours	1.5×10^7
18P15M	18	Oxygenation for 3 hours	1.5×10^7
18P18M	18	Oxygenation for 3 hours	1.8×10^7
24P15M	24	Oxygenation for 3 hours	1.5×10^7
24P24M	24	Oxygenation for 3 hours	2.4×10^7

4.2.1 The impact of wort gravity and pitching rate on fermentation progression

4.2.1.1 The effect of wort gravity and pitching rate on sugar utilisation

Measurement of wort gravity (Section 2.7.1) is commonly used to reflect the utilisation of wort sugars during brewing fermentation (Boulton & Quain, 2001), and the time to reach 80 % ADF (apparent degree of fermentation) is one of the key attributes in determining fermentation efficiency (Erten, *et al.*, 2007, Dekoninck, *et al.*, 2012, Gibson, *et al.*, 2013). Consequently fermentation curves were analysed and 80 % ADF was calculated to provide multiple indicators of fermentation efficiency.

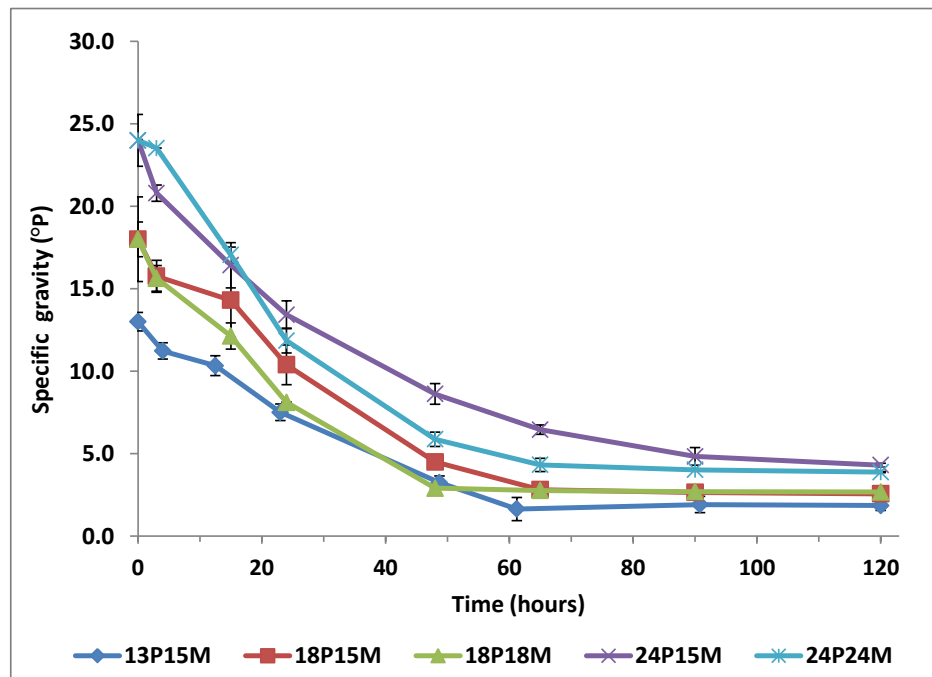
As expected, the change in specific gravity produced a sigmoidal curve during each set of fermentations (Figures 4.1-4.2). This pattern of change appeared to be conserved independent of the nature of the brewing yeast strain used. However, it was noticed that increasing the starting wort concentration resulted in a higher attenuation gravity regardless of yeast strain or pitching rate applied; 13P15M and 24P15M conditions showed the lowest and highest

attenuation gravity, respectively (Figures 4.1-4.2). Whilst similar attenuation gravities were obtained at 18P15M and 18P18M fermentations, 24P15M conditions resulted in elevated attenuation gravity compared to 24P24M and this was most pronounced in the ale strain NCYC1332 (Figure 4.2A). Additionally, with 24 °P fermentations using the four yeast strains, 24P15M conditions were found to display the slowest fermentation rate (Table 4.3). However a quicker attenuation could be achieved by increasing the initial cell density to 2.4×10^7 cells/mL (24P24M condition), as described previously. In contrast, at 18 °P fermentations, an accelerated fermentation rate could only be seen with strain Lager1 when comparing 18P18M and 18P15M fermentations (Table 4.3).

Table 4.3 Time taken to reach 80 % of ADF (apparent degree of fermentation) under different fermentation conditions. Data represent the mean of triplicate samples \pm standard deviation from independent tests.

<i>Yeast strain</i>	<i>Time taken to reach 80 % of ADF</i>				
	<i>13P15M</i>	<i>18P15M</i>	<i>18P18M</i>	<i>24P15M</i>	<i>24P24M</i>
Lager1	52.2 \pm 0.1	54.0 \pm 0.1	43.0 \pm 0.0	90.1 \pm 0.1	56.2 \pm 0.1
W34/70	46.2 \pm 0.4	46.1 \pm 0.2	51.2 \pm 0.4	97.0 \pm 0.2	53.3 \pm 0.5
NCYC1332	45.3 \pm 0.2	44.0 \pm 0.1	47.1 \pm 0.3	81.1 \pm 0.1	46.5 \pm 0.2
M2	39.0 \pm 0.1	39.3 \pm 0.3	39.2 \pm 0.1	85.2 \pm 0.2	50.1 \pm 0.3

(A)



(B)

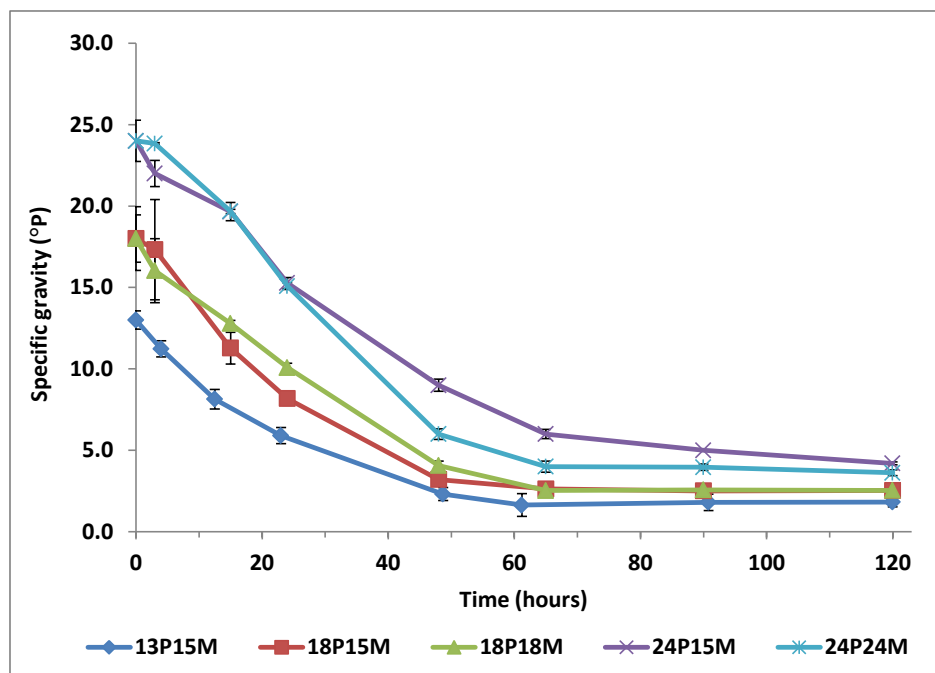
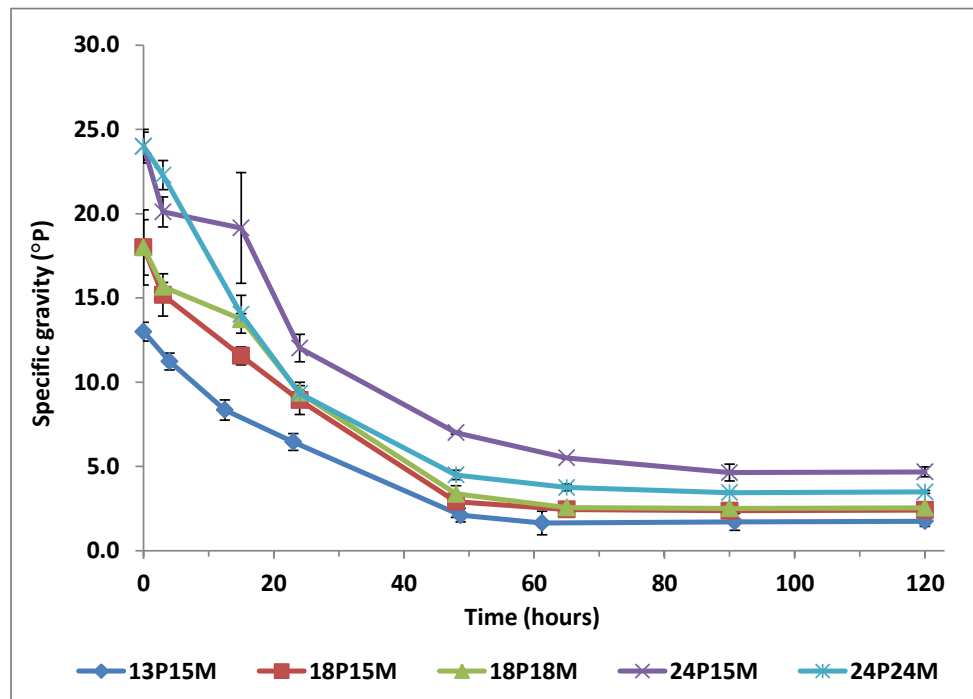


Figure 4.1 Decrease in specific gravity during fermentations using lager yeast strains Lager1 (A) and W34/70 (B) as a function of time. Gravity was assessed using a DMA 4500 density meter. Samples were taken at approximately 0, 3, 15, 24, 48, 65, 90 and 120 hours and yeast was removed through centrifugation. Data points represent the mean of triplicate samples, with error bars showing the standard deviation.

(A)



(B)

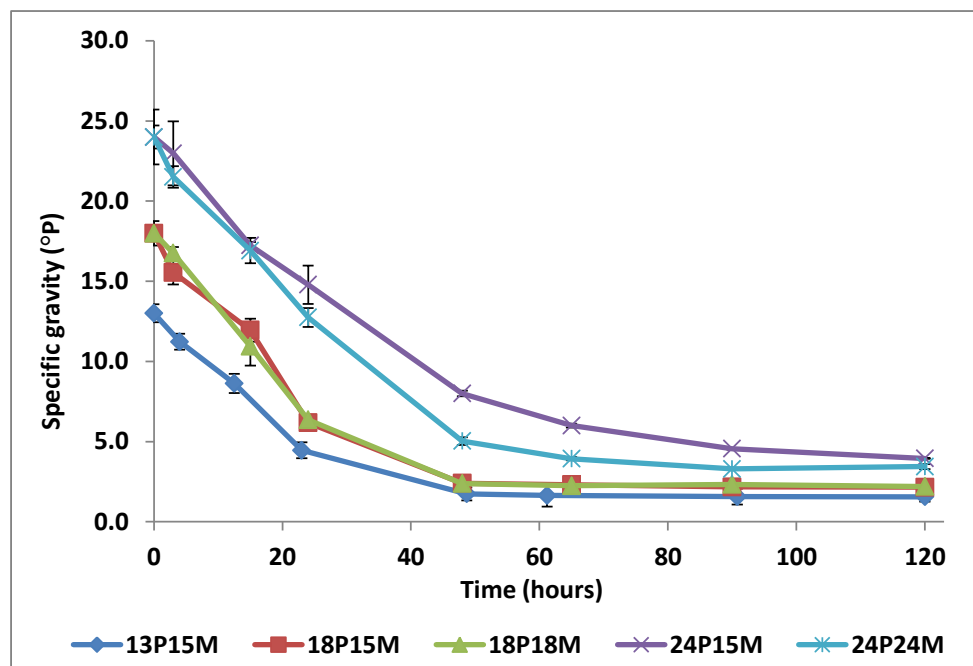


Figure 4.2 Decrease in specific gravity during fermentations using ale yeast strains NCYC1332 (A) and M2 (B) as a function of time. Gravity was assessed using a DMA 4500 density meter. Samples were taken at approximately 0, 3, 15, 24, 48, 65, 90 and 120 hours and yeast was removed through centrifugation. Data points represent the mean of triplicate samples, with error bars showing the standard deviation.

4.2.1.2 The effect of wort gravity and pitching rate on assimilation of individual carbohydrate

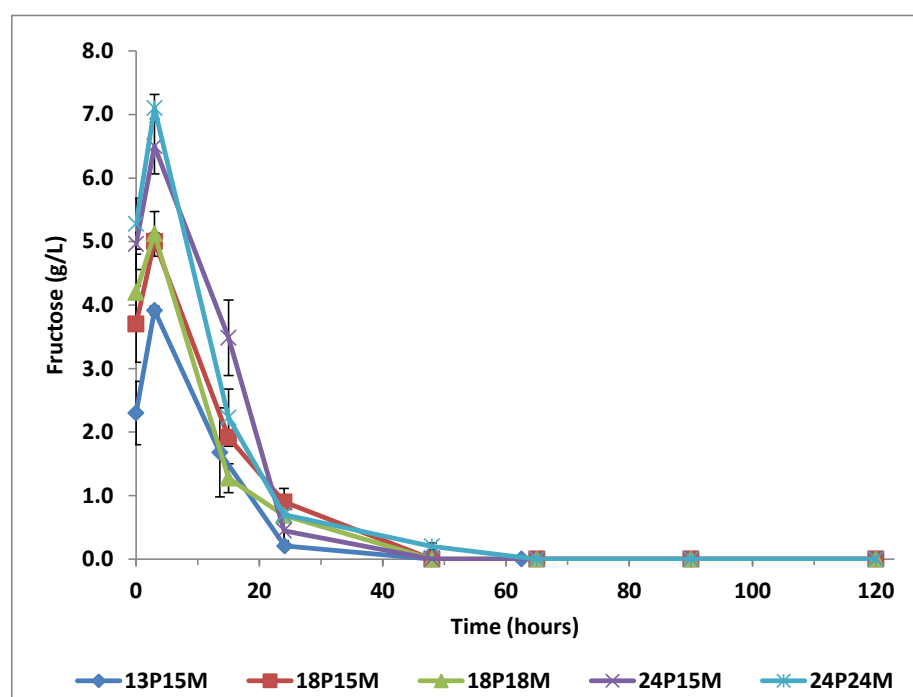
Concentrations of each carbohydrate in wort and beer samples were determined using high-performance liquid chromatography (HPLC) (Section 2.7.2). Although sucrose was present at low levels in the wort before pitching, it could not be detected in significant amounts during analysis. This is most likely because sucrose was hydrolysed prior to the first sampling point at approximately 3 hours, leading to a transient increase of fructose and glucose, a widely reported phenomenon (Boulton & Quain, 2001, Briggs, *et al.*, 2009).

In each of the five studied conditions, uptake of sugars was observed to occur in the same order, with fructose and glucose taken up first followed by high utilisation of maltose and maltotriose (Figures 4.3-4.10). However, the rate of assimilation differed between yeast strains. The concentrations of fructose (Figures 4.3-4.4) and glucose (Figures 4.5-4.6) increased initially (within 3 hours) due to sucrose hydrolysis, followed by consumption within a short time period thereafter. Utilisation of these sugars was observed to be quicker at 24P24M and 18P18M conditions compared to 24P15M and 18P15M conditions, respectively. Despite this, the concentrations of these sugars dropped to below 1 g/L within 24 hours post-pitching and they were completely consumed before 60 hours, irrespective of starting gravity (Figures 4.3-4.6).

Whilst small decreases in maltose concentration (Figures 4.7-4.8) were observed within the first 24 hours, the greatest utilisation occurred after this time point for all fermentations. The rate of consumption was observed to be related to pitching rate, with 24P24M and 18P18M fermentations showing higher assimilation rates than 24P15M and 18P15M fermentations, respectively. However, the final concentrations of maltose present were statistically the same at the end of the fermentations, irrespective of yeast strains. In a similar fashion to maltose,

consumption of maltotriose (Figures 4.9-4.10) was initially characterised by a small reduction, followed by a more dramatic utilisation approximately 15 hours post-pitching. The assimilation was quicker at 24P24M and 18P18M compared to 24P15M and 18P15M fermentations, respectively. In addition, faster maltotriose utilisation was observed with fermentations conducted using lager strains (Figure 4.9) than using ale strains (Figure 4.10), confirming previous reports that lager strains are able to utilize maltotriose more rapidly than ale yeast (Stewart, *et al.*, 1995). The rate of maltotriose uptake began to slow down approximately 70 hours post-pitching and ultimately a proportion of residual (unfermented) sugars remained present. For fermentations conducted with a pitching rate of 1.5×10^7 viable cells/mL, residual maltotriose levels were observed to increase with elevated wort gravity, with NCYC1332 showing the highest concentration of residual maltotriose (7.90 ± 0.33 g/L) at 24P15M condition (Figure 4.10A). Nevertheless, the uptake of maltotriose was greatly improved at 24P24M conditions compared to 24P15M conditions, irrespective of the yeast strain applied.

(A)



(B)

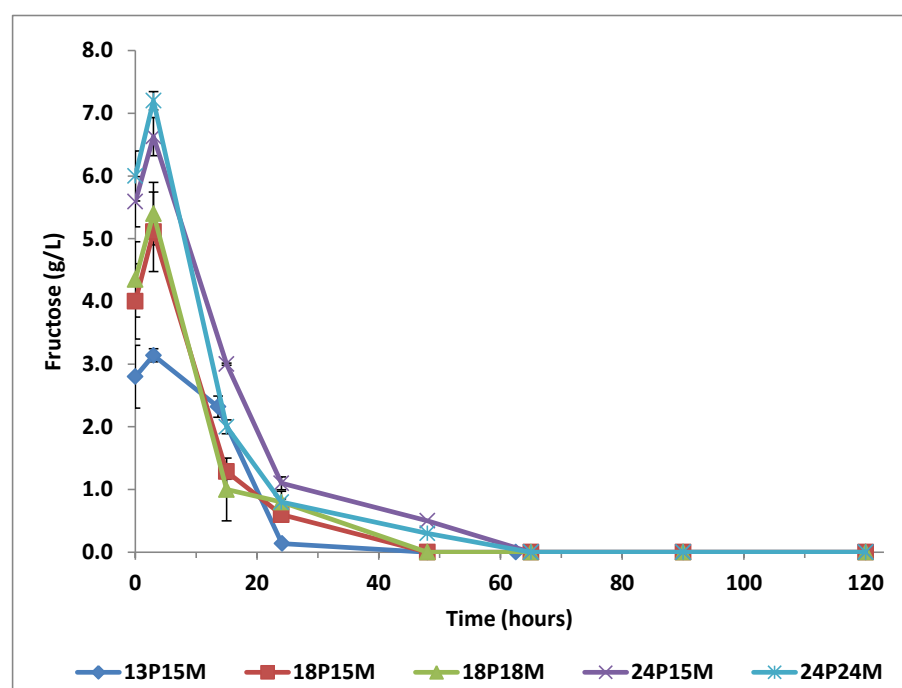
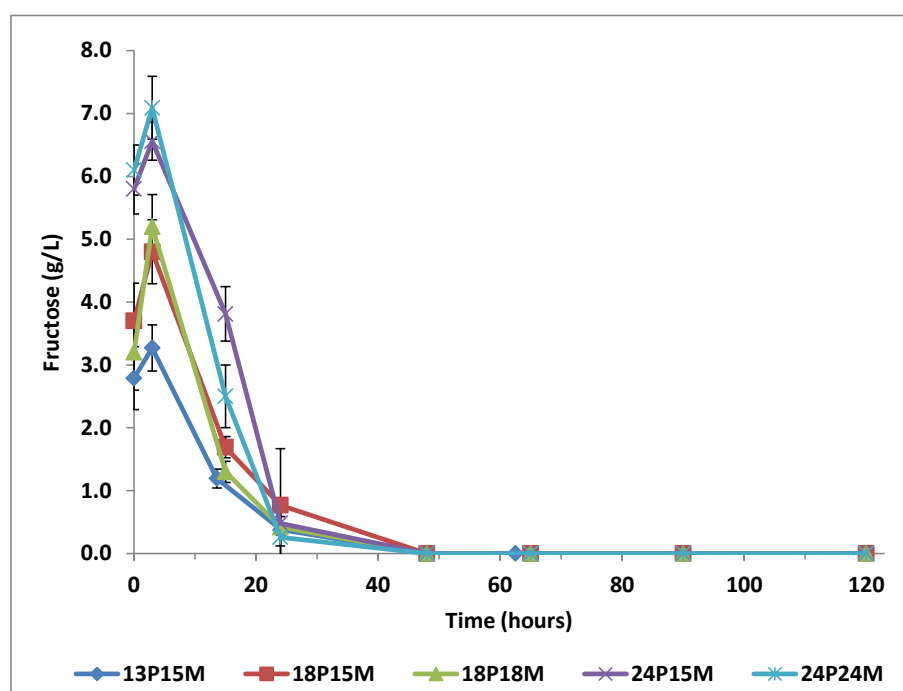


Figure 4.3 Concentration of fructose during fermentations using lager yeast strains Lager1 (A) and W34/70 (B) as a function of time, assessed using HPLC analysis. Samples were taken at approximately 0, 3, 15, 24, 48, 65, 90 and 120 hours and yeast was removed through centrifugation. Data points represent the mean of triplicate samples, with error bars showing the standard deviation.

(A)



(B)

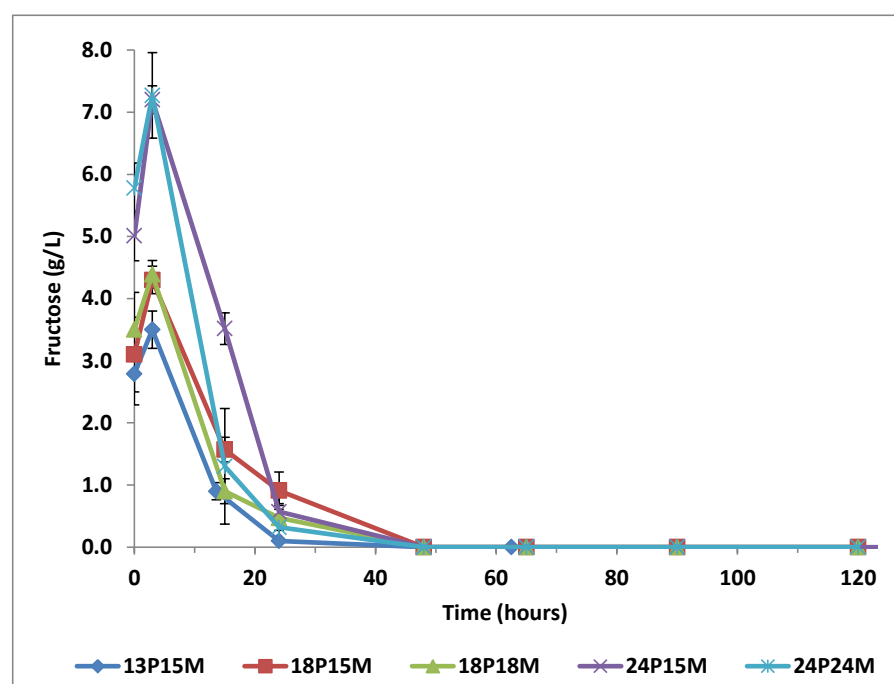
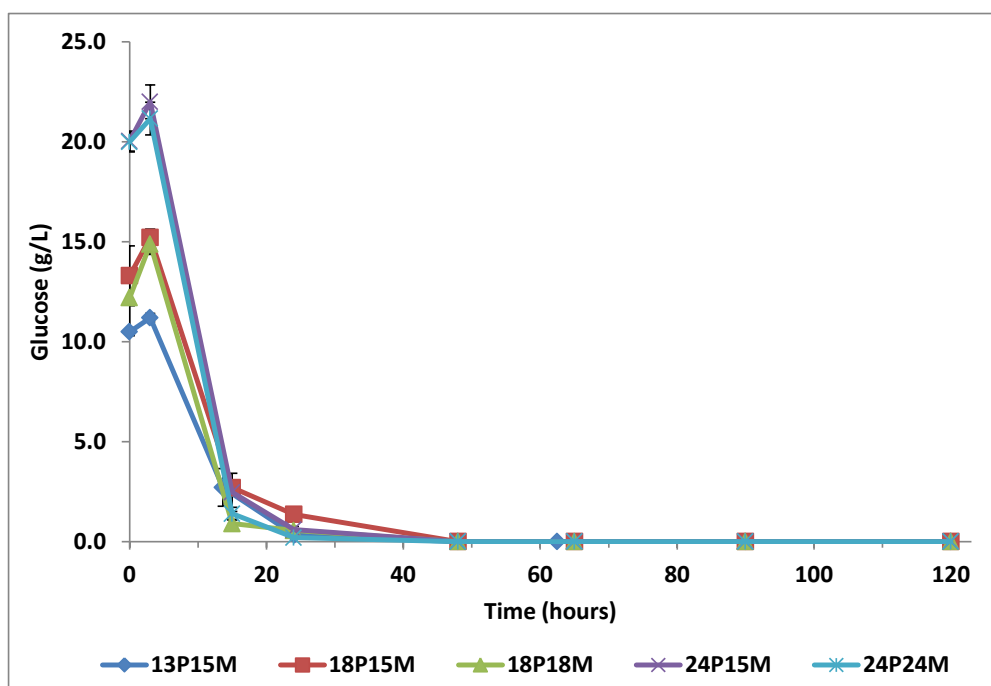


Figure 4.4 Concentration of fructose during fermentations using ale yeast strains NCYC1332 (A) and M2 (B) as a function of time, assessed using HPLC analysis. Samples were taken at approximately 0, 3, 15, 24, 48, 65, 90 and 120 hours and yeast was removed through centrifugation. Data points represent the mean of triplicate samples, with error bars showing the standard deviation.

(A)



(B)

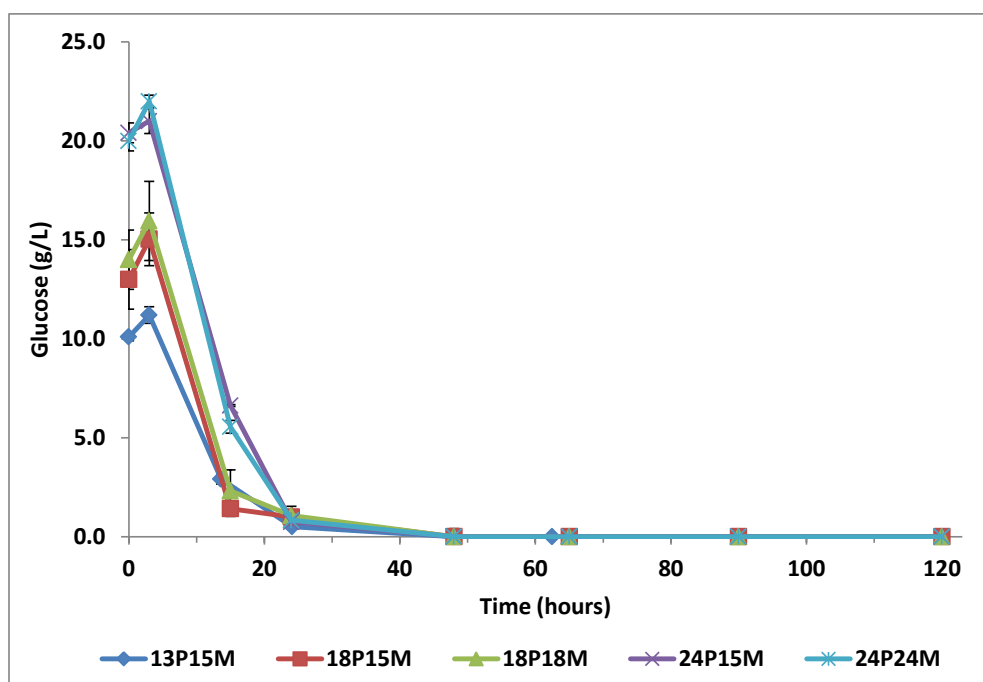
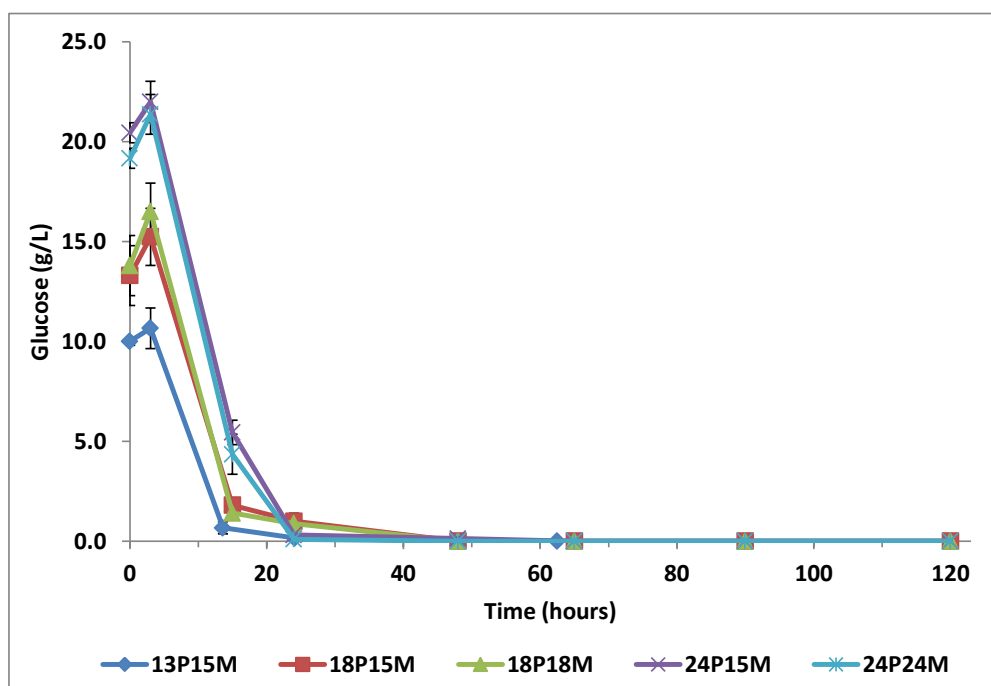


Figure 4.5 Concentration of glucose during fermentations using lager yeast strains Lager1 (A) and W34/70 (B) as a function of time, assessed using HPLC analysis. Samples were taken at approximately 0, 3, 15, 24, 48, 65, 90 and 120 hours and yeast was removed through centrifugation. Data points represent the mean of triplicate samples, with error bars showing the standard deviation.

(A)



(B)

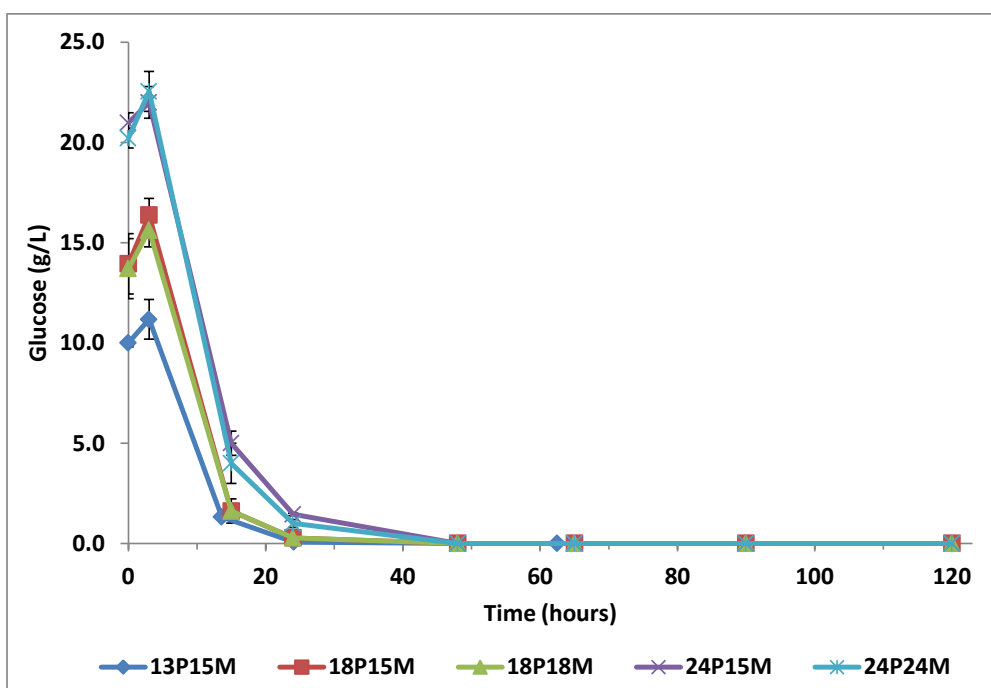
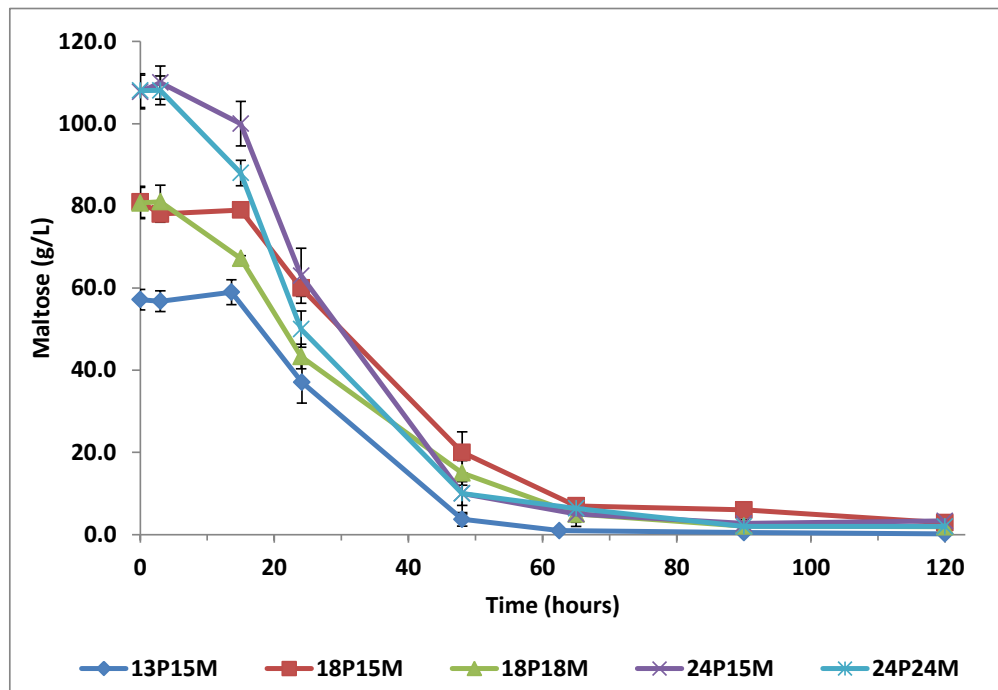


Figure 4.6 Concentration of glucose during fermentations using ale yeast strains NCYC1332 (A) and M2 (B) as a function of time, assessed using HPLC analysis. Samples were taken at approximately 0, 3, 15, 24, 48, 65, 90 and 120 hours and yeast was removed through centrifugation. Data points represent the mean of triplicate samples, with error bars showing the standard deviation.

(A)



(B)

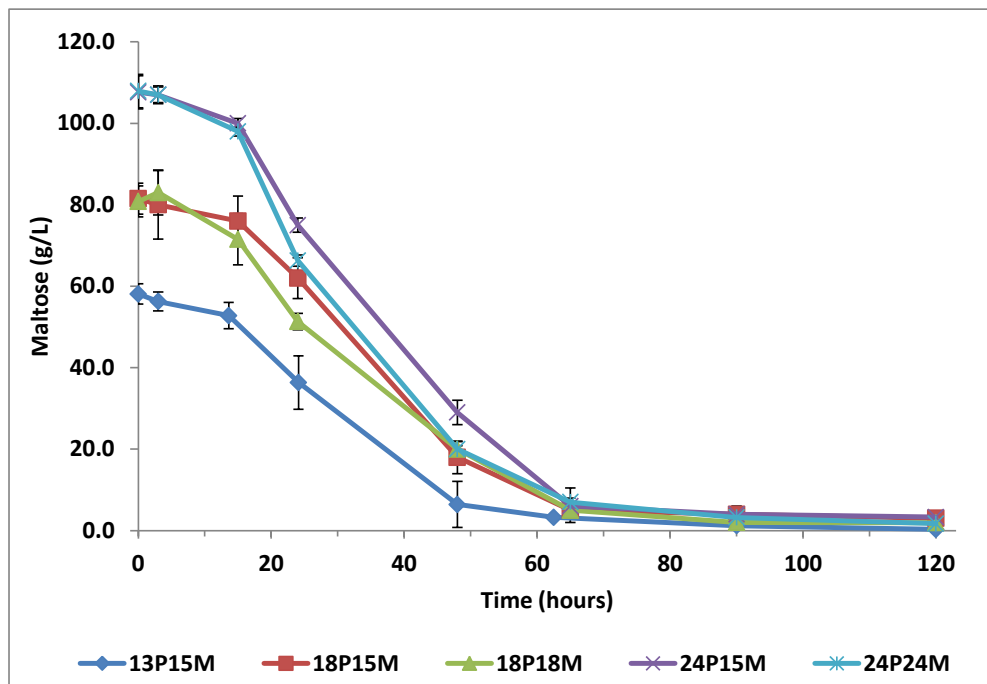
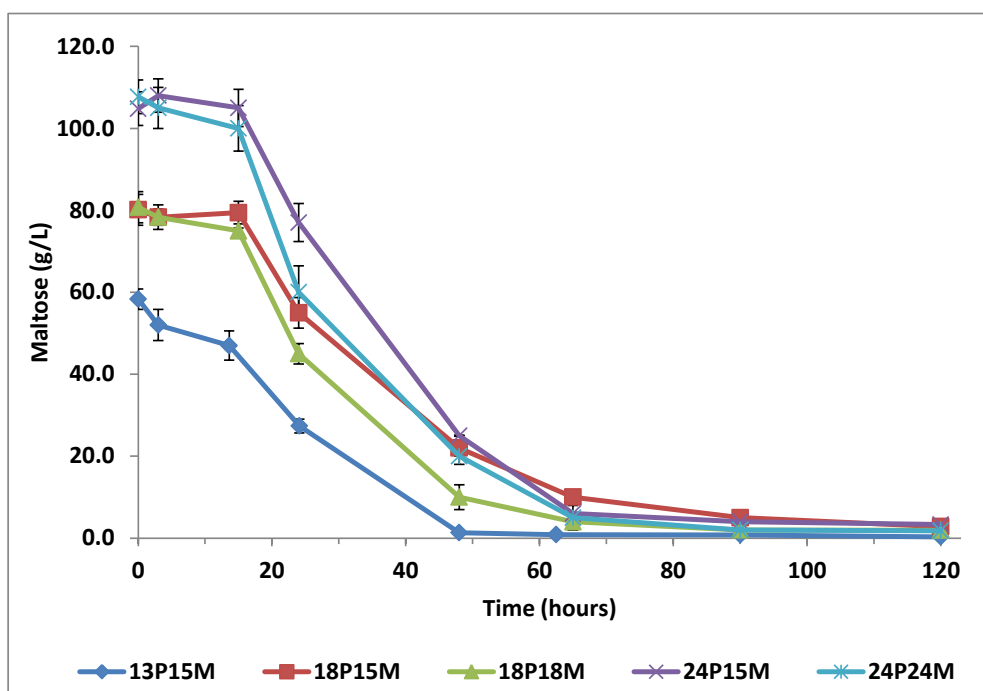


Figure 4.7 Concentration of maltose during fermentations using lager yeast strains Lager1 (A) and W34/70 (B) as a function of time, assessed using HPLC analysis. Samples were taken at approximately 0, 3, 15, 24, 48, 65, 90 and 120 hours and yeast was removed through centrifugation. Data points represent the mean of triplicate samples, with error bars showing the standard deviation.

(A)



(B)

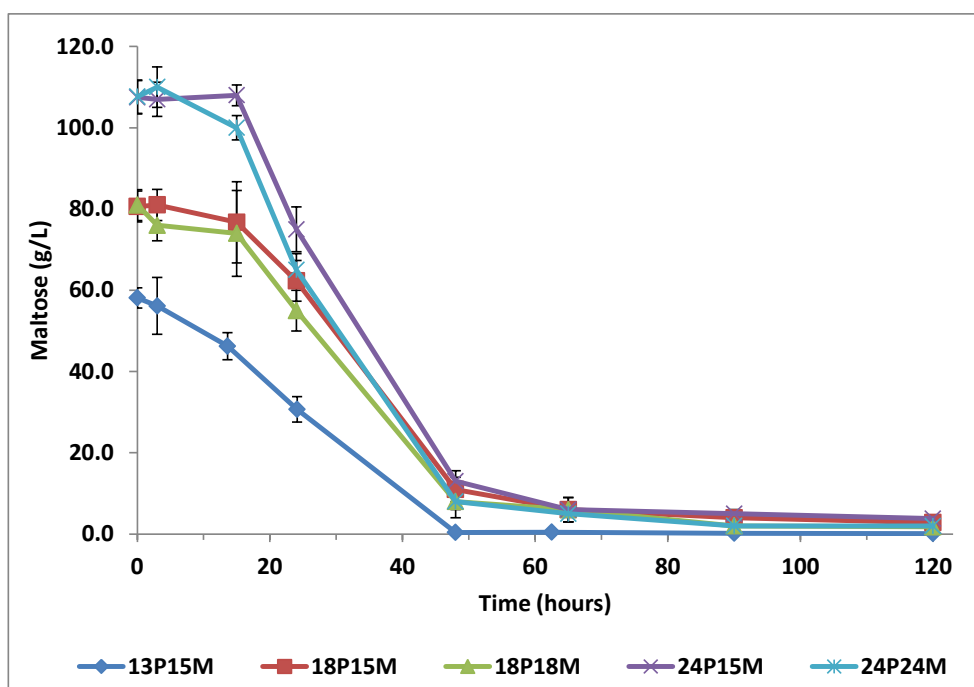


Figure 4.8 Concentration of maltose during fermentations using ale yeast strains NCYC1332 (A) and M2 (B) as a function of time, assessed using HPLC analysis. Samples were taken at approximately 0, 3, 15, 24, 48, 65, 90 and 120 hours and yeast was removed through centrifugation. Data points represent the mean of triplicate samples, with error bars showing the standard deviation.

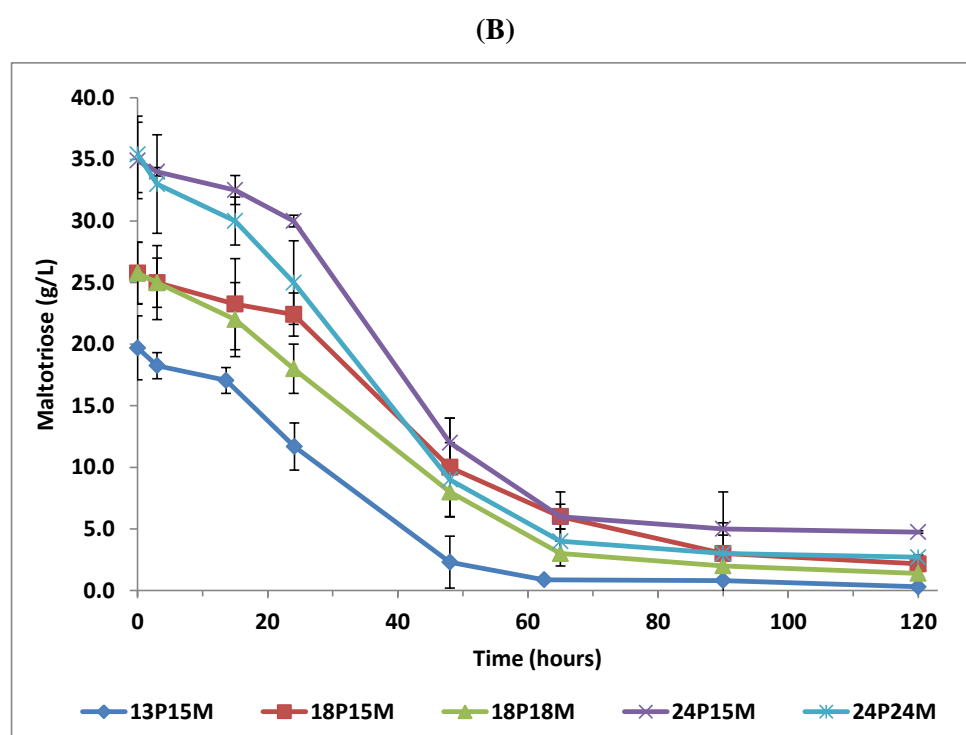
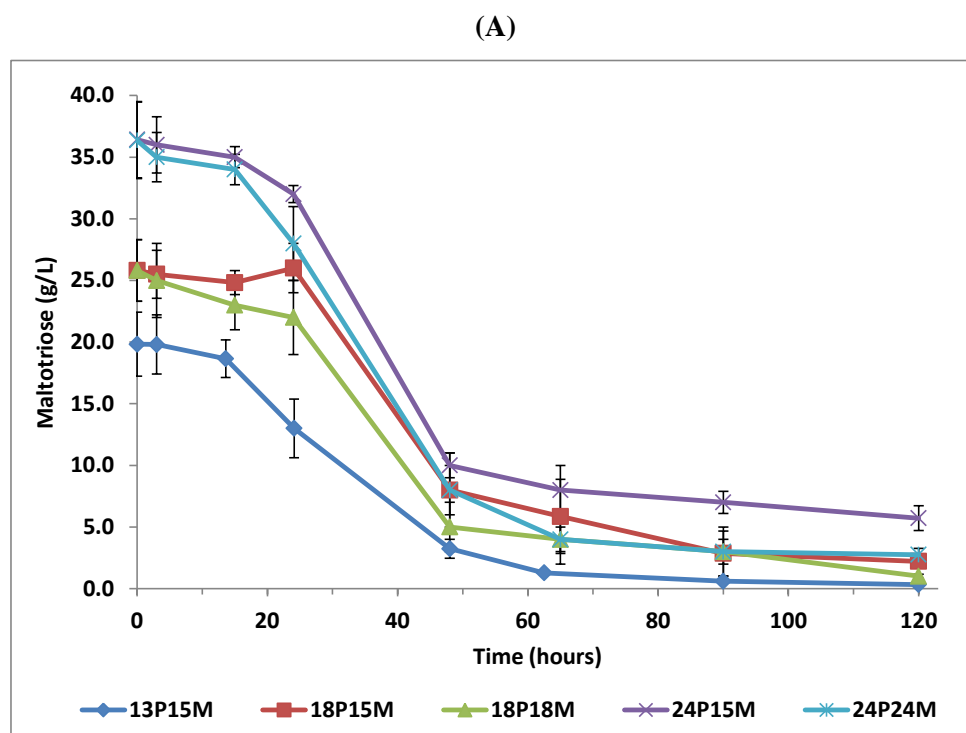


Figure 4.9 Concentration of maltotriose during fermentations using lager yeast strains Lager1 (A) and W34/70 (B) as a function of time, assessed using HPLC analysis. Samples were taken at approximately 0, 3, 15, 24, 48, 65, 90 and 120 hours and yeast was removed through centrifugation. Data points represent the mean of triplicate samples, with error bars showing the standard deviation.

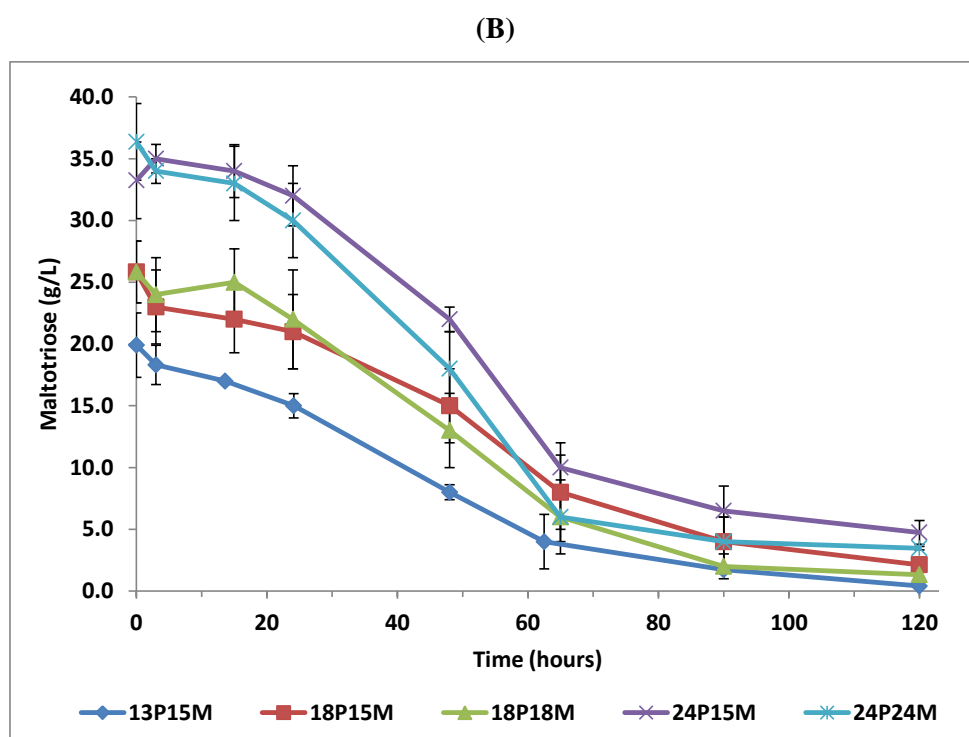
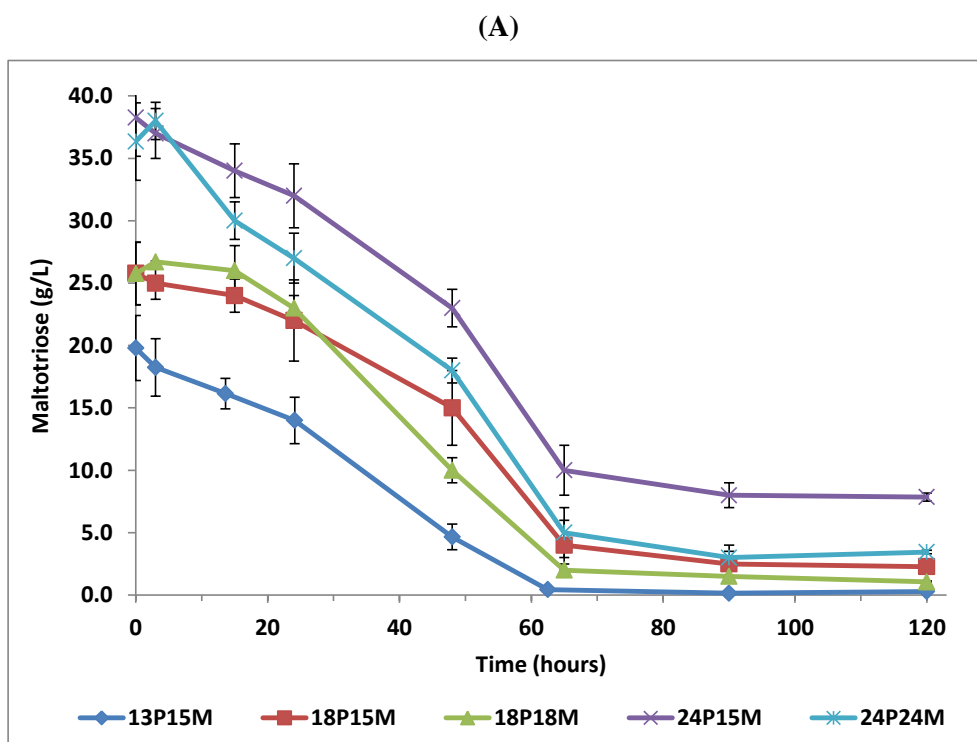


Figure 4.10 Concentration of maltotriose during fermentations using ale yeast strains NCYC1332 (A) and M2 (B) as a function of time, assessed using HPLC analysis. Samples were taken at approximately 0, 3, 15, 24, 48, 65, 90 and 120 hours and yeast was removed through centrifugation. Data points represent the mean of triplicate samples, with error bars showing the standard deviation.

4.2.1.3 The effect of wort gravity and pitching rate on ethanol production

Ethanol is the major product of carbohydrate dissimilation in brewing yeast, occurring via the fermentative pathway. In this study the concentration of ethanol in wort and beer samples was determined using HPLC (Section 2.7.4) and expressed in g/L.

During the course of fermentation, the formation of ethanol displayed a profile with progressive changes in the rate of production (Figures 4.11-4.12). The fermentations achieved maximum ethanol concentration at approximately 65 hours post-pitching in all instances, except for 24P15M conditions, which showed an increase in ethanol until 120 hours. Additionally, whilst ethanol production was statistically the same for each strain at every point between 18P15M and 18P18M conditions, a more rapid ethanol yield was observed in 24P24M when compared to 24P15M conditions. In general, final ethanol yield was found to increase in proportion to the original wort gravity, irrespective of yeast type (Table 4.4). It was also observed that 18P15M and 18P18M conditions produced similar amount of ethanol, whereas 24P24M resulted in significantly ($P < 0.05$) higher levels of ethanol compared to 24P15M fermentations for each of the four yeast strains (Table 4.4).

Table 4.4 Concentrations of ethanol at the end of fermentations, assessed using HPLC. Data represent the mean of triplicate samples \pm standard deviation from independent tests.

	<i>Final ethanol yield (g/L)</i>				
	<i>13P15M</i>	<i>18P15M</i>	<i>18P18M</i>	<i>24P15M</i>	<i>24P24M</i>
Lager1	43.9 \pm 0.7	58.5 \pm 0.5	60.1 \pm 1.1	77.5 \pm 0.5	85.0 \pm 0.8
W34/70	44.0 \pm 0.9	58.8 \pm 0.5	60.0 \pm 0.8	80.8 \pm 0.5	84.5 \pm 0.7
NCYC1332	43.0 \pm 1.0	54.9 \pm 0.8	57.3 \pm 1.1	72.4 \pm 0.8	79.5 \pm 0.7
M2	43.5 \pm 0.6	57.0 \pm 0.8	59.3 \pm 0.4	78.4 \pm 1.3	82.5 \pm 0.8

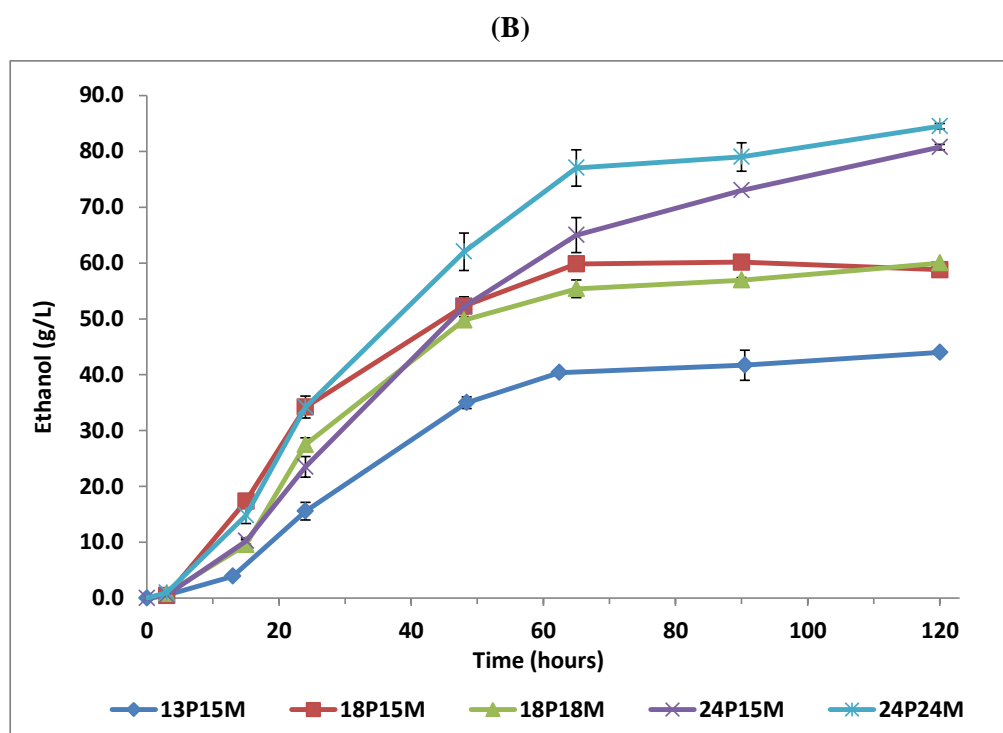
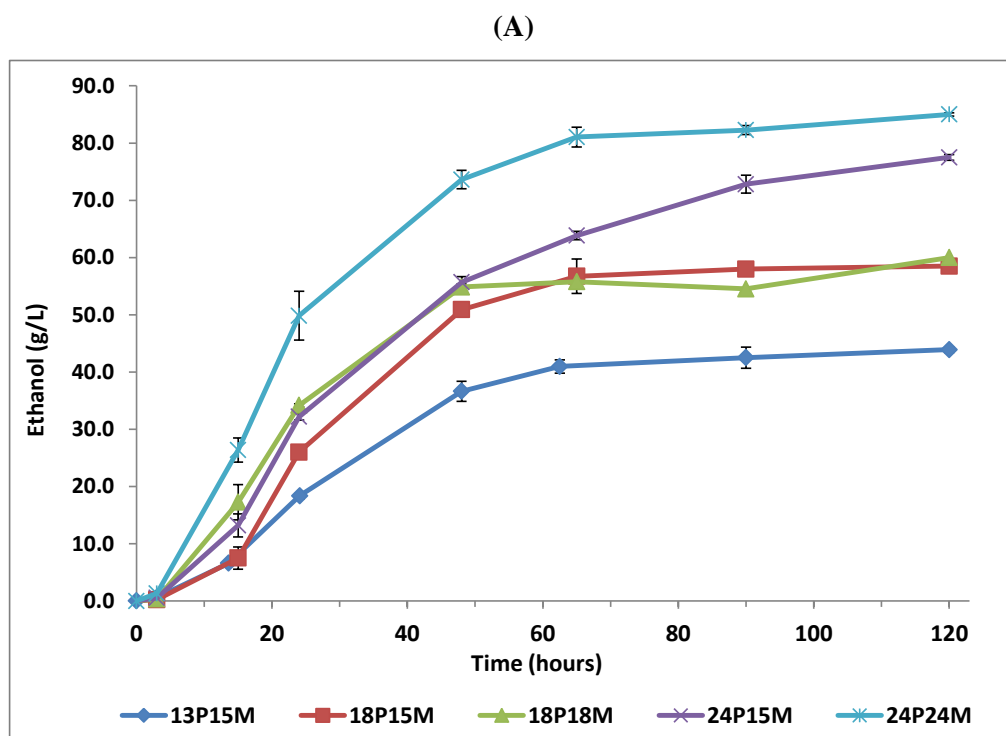


Figure 4.11 Concentrations of ethanol during fermentations using lager strains Lager1 (A) and W34/70 (B) as a function of time, assessed using HPLC analysis. Samples were taken at approximately 0, 3, 15, 24, 48, 65, 90 and 120 hours and yeast was removed through centrifugation. Data points represent the mean of triplicate samples, with error bars showing the standard deviation.

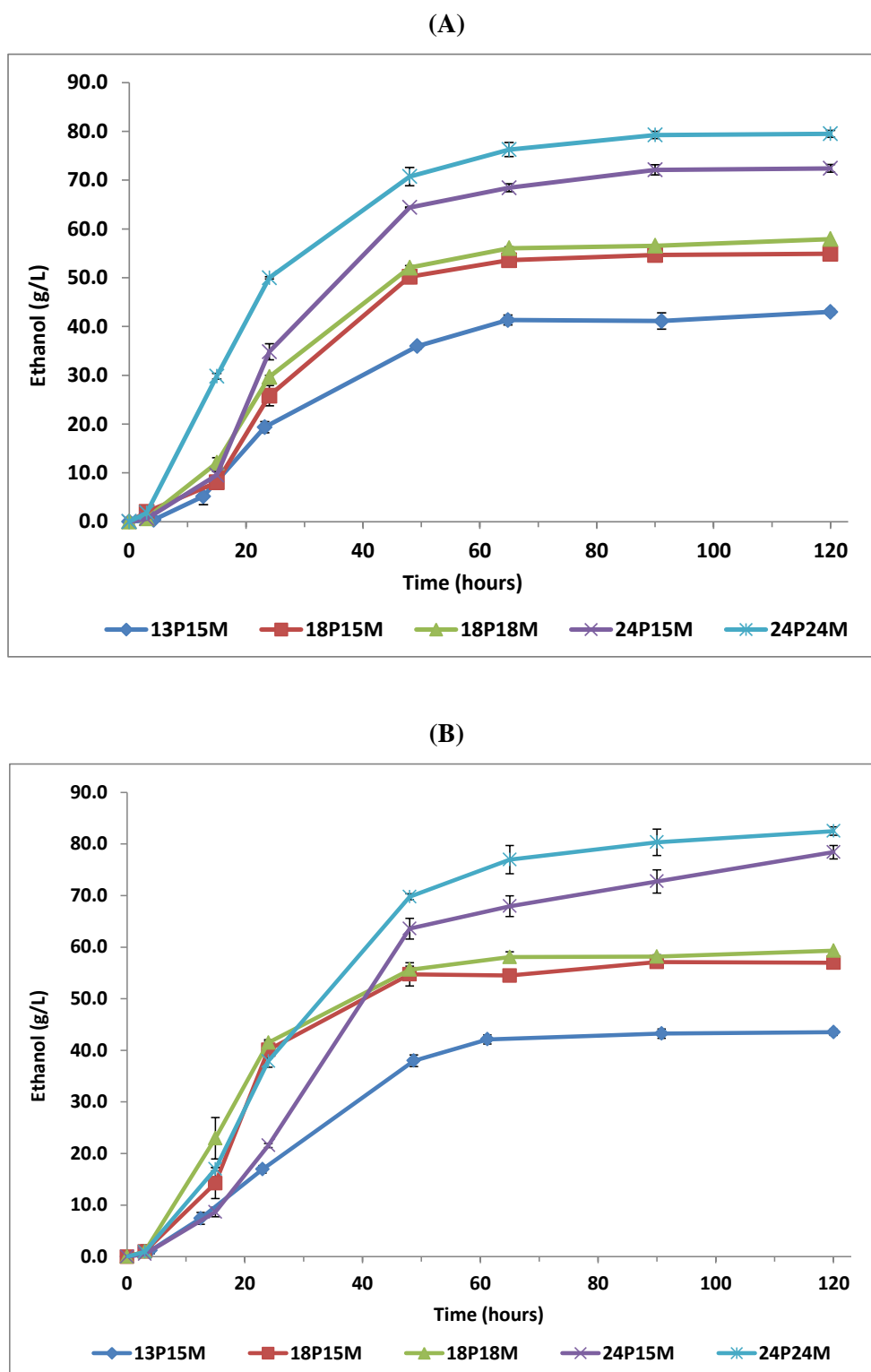


Figure 4.12 Concentrations of ethanol during fermentations using ale strains NCYC1332 (A) and M2 (B) as a function of time, assessed using HPLC analysis. Samples were taken at approximately 0, 3, 15, 24, 48, 65, 90 and 120 hours and yeast was removed through centrifugation. Data points represent the mean of triplicate samples, with error bars showing the standard deviation.

4.2.1.4 The effect of wort gravity and pitching rate on carbon dioxide evolution

During the course of fermentative metabolism, carbon dioxide is produced in equimolar amounts to ethanol (Boulton & Quain, 2001). In the current study, a constant agitation (350 rpm) and a gas outlet port within the fermenter (Section 2.6.3) allowed the release of CO₂ during fermentation. In order to determine CO₂ production, the weight of triplicate fermenters was recorded at appropriate intervals, and the concentration of CO₂ was determined by subtracting the real-time weight from the original (0 hour) weight of corresponding fermenters and expresses in g/L.

The profiles of CO₂ evolution for each set of fermentations are depicted in Figures 4.13-4.14. For fermentations with a pitching rate of 1.5×10^7 cells/mL, 13P15M conditions exhibited the slowest fermentation progression. Although similar CO₂ evolution patterns were observed under 18P15M and 24P15M conditions until 48 hours post-pitching, after this point the 24P15M fermentations resulted in higher CO₂ evolution values than at 18P15M. This was true for all of the yeast strains applied. In addition, although CO₂ evolution was statistically the same between 18P18M and 18P15M conditions at every sampling point, fermentation progression was quicker at 24P24M than 24P15M conditions for each of the four yeast strains. Similarly, whilst the mean values for CO₂ evolution at the end of fermentation were found to be statistically identical for 18P18M and 18P15M, a much bigger difference was observed when comparing 24P24M and 24P15M conditions, with the higher pitching rate showing a greater CO₂ production, potentially indicating incomplete sugar conversion under 24P15M conditions. Despite this observation, total CO₂ evolution increased with the original wort gravity irrespective of yeast strain. Unsurprisingly, the concentration of CO₂ at each sampling point was consistent with that of ethanol previously reported (Section 4.2.1.3), with a correlation coefficient of >0.99 in each instance (Table 4.5), indicating that both techniques are valuable as measures of fermentation progression.

Table 4.5 Correlation coefficient analysis between ethanol yield and carbon dioxide evolution at each sampling point, assessed using Microsoft Excel 2010 software.

<i>Test conditions</i>	Yeast strains			
	Lager1	W34/70	NCYC1332	M2
13P15M	0.996	0.996	0.998	0.998
18P15M	0.998	0.996	0.994	0.991
18P18M	0.996	0.998	0.999	0.998
24P15M	0.999	0.996	0.997	0.992
24P24M	0.999	0.998	0.999	0.998

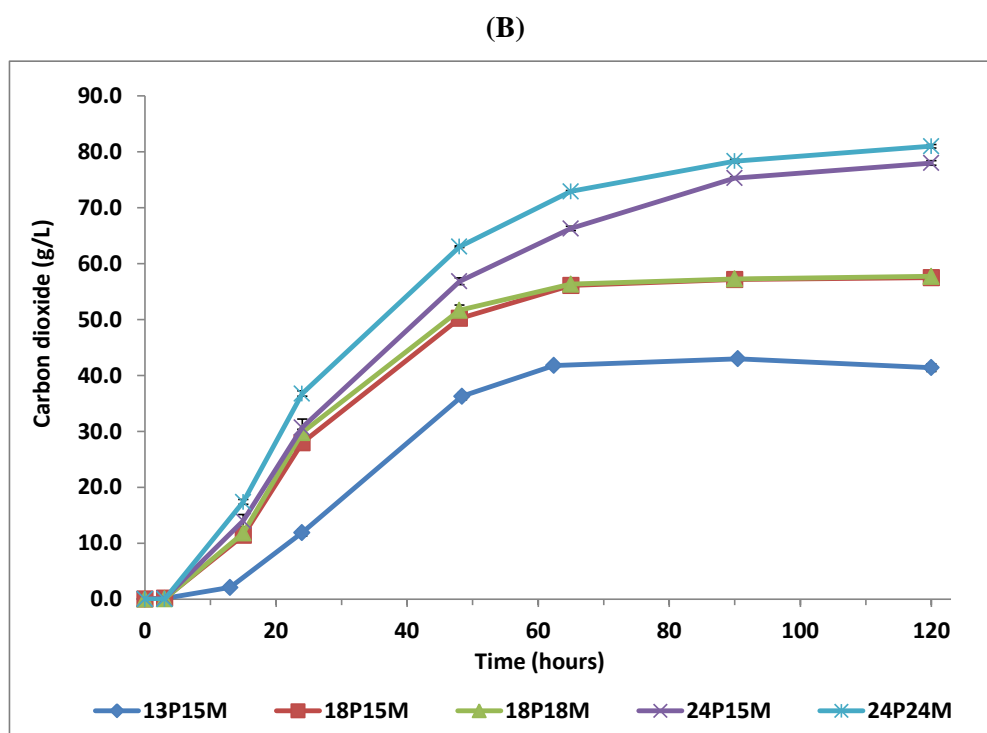
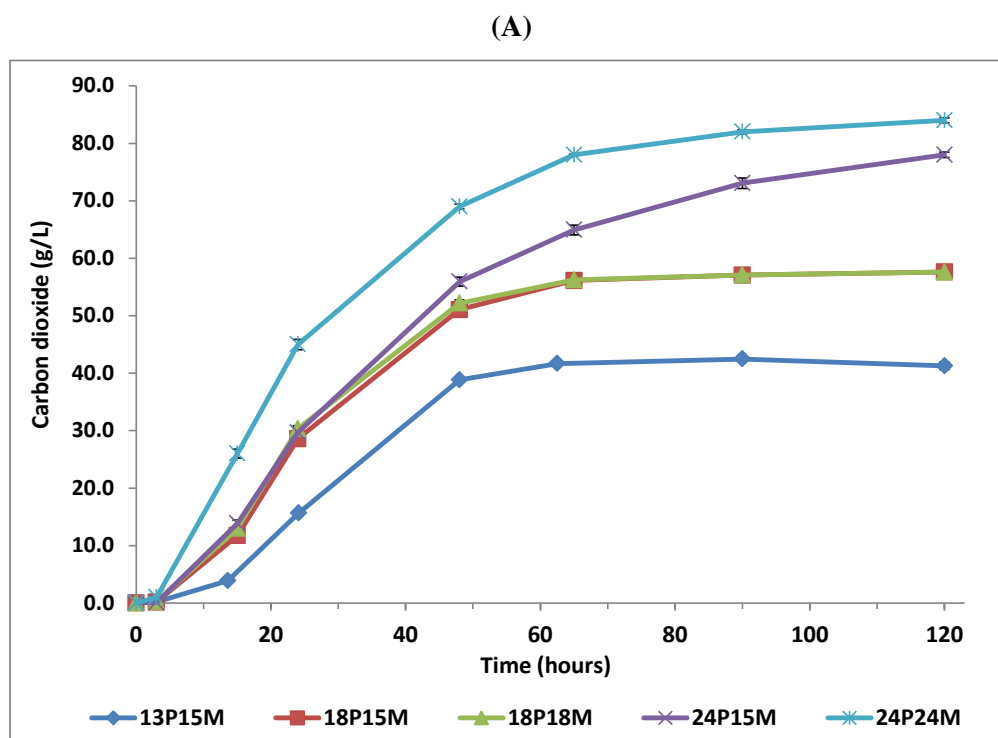


Figure 4.13 Concentrations of carbon dioxide evolved during fermentations using lager strains Lager1 (A) and W34/70 (B) as a function of time, based on the weight loss of fermentation vessels. Measurements were conducted at approximately 0, 3, 15, 24, 48, 65, 90 and 120 hours. Data points represent the mean of triplicate samples, with error bars showing the standard deviation.

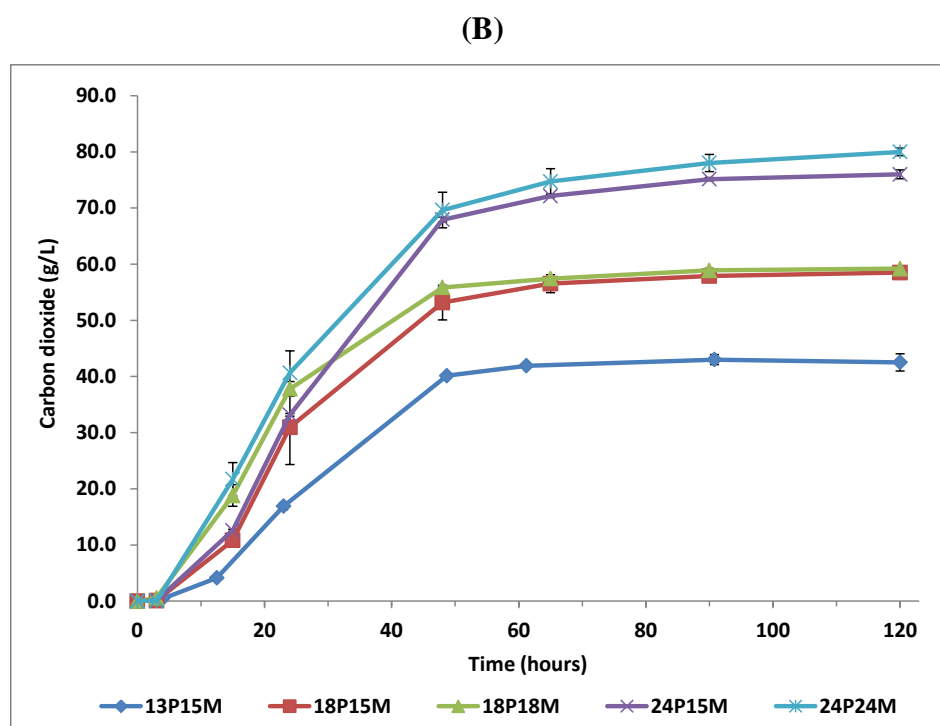
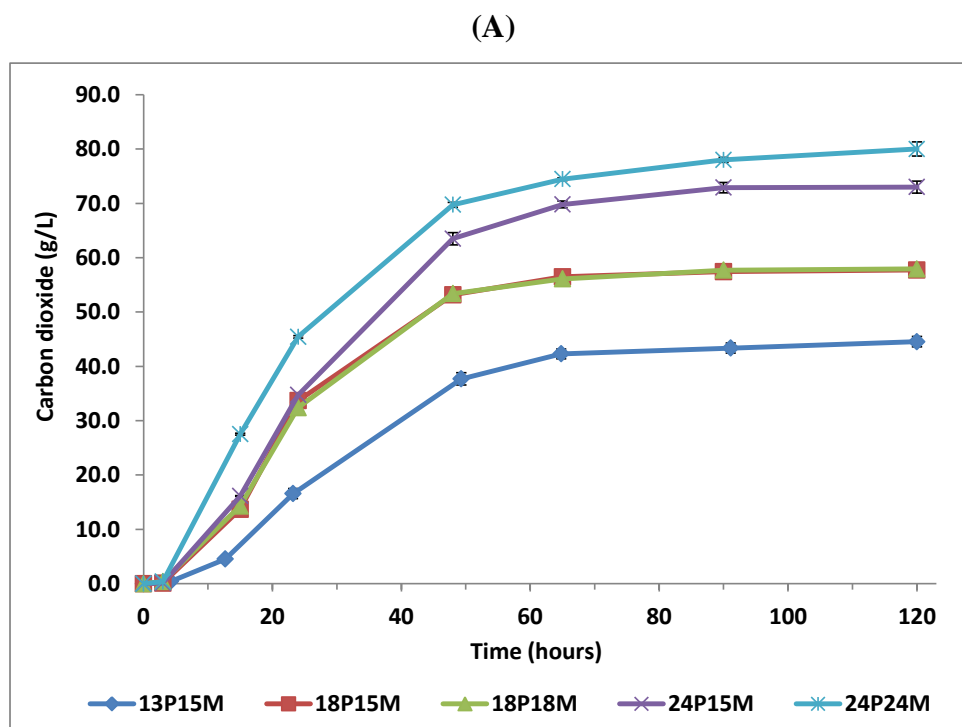


Figure 4.14 Concentrations of carbon dioxide evolved during fermentations using ale strains NCYC1332 (A) and M2 (B) as a function of time, based on the weight loss of fermentation vessels. Measurements were conducted at approximately 0, 3, 15, 24, 48, 65, 90 and 120 hours. Data points represent the mean of triplicate samples, with error bars showing the standard deviation.

4.2.1.5 The effect of wort gravity and pitching rate on flavour-active compounds

Higher alcohols and esters are two major groups of flavour-active compounds, and their levels of production have a significant effect on beer taste and aroma (Pires, *et al.*, 2014). In order to compare the production of flavour compounds at different gravities, each final beer was analysed using headspace GC-MS (Section 2.7.6). In each instance, values were normalized based on a standard ethanol content in beer (5 %, v/v) and results are summarized in Tables 4.6-4.9. It should be noted that 13 °P fermentations were not used as a comparison against 18 °P and 24 °P brews in terms of flavour synthesis, since the latter were fermented based on un-hopped wort whereas the former was conducted using hopped wort (Section 2.6.1).

In comparing 18P15M and 24P15M conditions, it can be seen that the concentrations of total higher alcohols were significantly ($P < 0.05$) higher at 18P15M fermentations, regardless of the yeast strain used. Additionally, elevated levels of total higher alcohols were also observed in fermentations using ale strains (Tables 4.8-4.9) when compared to lager strains (Tables 4.6-4.7), irrespective of pitching rate. The largest contributor appeared to be isobutanol, produced to a particularly high concentration in ale strains. In contrast, the concentrations of amyl alcohol (2-methyl-1-butanol) and isoamyl alcohol (3-methyl-1-butanol), combined as ‘total amyl alcohol’ (since their peaks were not separated well in the chromatography), was not influenced by wort gravity or pitching rate, but remained conserved between yeast strains. As for ester production, ethyl acetate, isoamyl acetate and other ethyl esters including ethyl propionate, ethyl butyrate and ethyl caproate were detected. With increased wort gravity (comparing 24P15M to 18P15M), the most significant change was the increased concentration of ethyl acetate irrespective of yeast strain. Apart from this, starting wort gravity and pitching rate did not have a profound effect on ester formation in any of the four strains studied.

Table 4.6 Concentrations of flavour-active compounds in final beers derived from different fermentations using lager yeast Lager1, assessed using headspace GC-MS analysis. Data points represent the mean of triplicate samples \pm standard deviation from independent tests ^a

	<i>Fermentation conditions</i>				
	<i>13P15M</i>	<i>18P15M</i>	<i>18P18M</i>	<i>24P15M</i>	<i>24P24M</i>
<i>Higher alcohols (mg/L)</i>					
<i>Isobutanol</i>	32.38 \pm 3.14	40.28 \pm 2.70	31.39 \pm 3.78	30.44 \pm 3.07	30.63 \pm 2.06
<i>Total amyl alcohol</i>	29.11 \pm 2.50	33.46 \pm 3.85	31.17 \pm 3.03	23.68 \pm 2.33	24.97 \pm 2.62
<i>Total higher alcohols</i>	61.49 \pm 5.62	73.75 \pm 5.36	62.56 \pm 6.66	54.12 \pm 5.13	55.60 \pm 4.40
<i>Esters (mg/L)</i>					
<i>Ethyl acetate</i>	13.84 \pm 4.83	19.55 \pm 0.56	20.61 \pm 1.57	26.27 \pm 1.33	23.41 \pm 3.46
<i>Isoamyl acetate</i>	2.78 \pm 0.45	4.26 \pm 1.75	3.26 \pm 0.96	3.72 \pm 0.51	2.93 \pm 0.86
<i>Ethyl propionate</i>	0.10 \pm 0.01	0.07 \pm 0.02	0.03 \pm 0.03	0.03 \pm 0.01	0.03 \pm 0.03
<i>Ethyl butyrate</i>	0.07 \pm 0.02	0.10 \pm 0.03	0.08 \pm 0.02	0.10 \pm 0.01	0.09 \pm 0.04
<i>Ethyl caproate</i>	0.38 \pm 0.11	0.27 \pm 0.08	0.35 \pm 0.07	0.25 \pm 0.02	0.23 \pm 0.06
<i>Total esters</i>	16.17 \pm 5.27	24.25 \pm 2.33	24.33 \pm 2.46	30.37 \pm 1.72	26.69 \pm 4.41

^a Values of flavour compounds was recalculated based on the standard ethanol content in beers (= 5 %, v/v).

Table 4.7 Concentrations of flavour-active compounds in final beers derived from different fermentations using lager yeast W34/70, assessed using headspace GC-MS analysis. Data points represent the mean of triplicate samples \pm standard deviation from independent tests ^a

	<i>Fermentation conditions</i>				
	<i>13P15M</i>	<i>18P15M</i>	<i>18P18M</i>	<i>24P15M</i>	<i>24P24M</i>
<i>Higher alcohols (mg/L)</i>					
<i>Isobutanol</i>	34.91 \pm 2.95	40.11 \pm 3.35	43.59 \pm 4.42	26.14 \pm 2.36	31.40 \pm 3.51
<i>Total amyl alcohol</i>	30.97 \pm 3.39	33.64 \pm 1.79	35.14 \pm 1.70	22.03 \pm 3.73	27.31 \pm 2.68
<i>Total higher alcohols</i>	65.88 \pm 5.30	73.74 \pm 3.89	78.73 \pm 5.11	48.18 \pm 5.10	58.72 \pm 5.68
<i>Esters (mg/L)</i>					
<i>Ethyl acetate</i>	19.73 \pm 3.26	25.76 \pm 1.02	25.14 \pm 3.98	29.61 \pm 2.52	32.10 \pm 2.00
<i>Isoamyl acetate</i>	2.90 \pm 1.68	5.70 \pm 0.51	4.90 \pm 0.54	3.77 \pm 1.22	5.35 \pm 1.10
<i>Ethyl propionate</i>	0.10 \pm 0.01	0.04 \pm 0.00	0.05 \pm 0.00	0.01 \pm 0.03	0.02 \pm 0.01
<i>Ethyl butyrate</i>	0.10 \pm 0.05	0.10 \pm 0.02	0.09 \pm 0.01	0.08 \pm 0.00	0.09 \pm 0.03
<i>Ethyl caproate</i>	0.58 \pm 0.17	0.28 \pm 0.06	0.44 \pm 0.04	0.25 \pm 0.24	0.23 \pm 0.04
<i>Total esters</i>	23.40 \pm 3.10	31.88 \pm 1.70	30.62 \pm 3.82	33.72 \pm 3.62	37.79 \pm 3.95

^a Values of flavour compounds was recalculated based on the standard ethanol content in beers (= 5 %, v/v).

Table 4.8 Concentrations of flavour-active compounds in final beers derived from different fermentations using ale yeast NCYC1332, assessed using headspace GC-MS analysis. Data points represent the mean of triplicate samples \pm standard deviation from independent tests ^a

	<i>Fermentation conditions</i>				
	<i>13P15M</i>	<i>18P15M</i>	<i>18P18M</i>	<i>24P15M</i>	<i>24P24M</i>
<i>Higher alcohols (mg/L)</i>					
<i>Isobutanol</i>	62.99 \pm 2.28	76.09 \pm 3.67	83.30 \pm 2.88	58.41 \pm 3.47	83.48 \pm 2.00
<i>Total amyl alcohol</i>	42.22 \pm 3.05	48.97 \pm 3.83	52.35 \pm 2.49	40.95 \pm 3.78	44.09 \pm 3.45
<i>Total higher alcohols</i>	105.21 \pm 5.30	125.06 \pm 6.48	135.65 \pm 4.24	99.36 \pm 6.58	127.57 \pm 5.02
<i>Esters (mg/L)</i>					
<i>Ethyl acetate</i>	22.89 \pm 0.44	25.44 \pm 3.85	23.23 \pm 3.44	32.09 \pm 2.34	26.51 \pm 2.86
<i>Isoamyl acetate</i>	4.01 \pm 0.54	6.85 \pm 1.93	5.37 \pm 1.21	7.22 \pm 1.36	6.85 \pm 1.25
<i>Ethyl propionate</i>	0.05 \pm 0.01	0.04 \pm 0.02	0.04 \pm 0.00	0.02 \pm 0.02	0.02 \pm 0.01
<i>Ethyl butyrate</i>	0.11 \pm 0.02	0.12 \pm 0.01	0.10 \pm 0.00	0.11 \pm 0.02	0.09 \pm 0.02
<i>Ethyl caproate</i>	0.44 \pm 0.13	0.31 \pm 0.05	0.27 \pm 0.03	0.26 \pm 0.03	0.15 \pm 0.09
<i>Total esters</i>	27.50 \pm 1.13	32.76 \pm 4.26	29.01 \pm 4.31	39.70 \pm 4.61	33.63 \pm 4.17

^a Values of flavour compounds was recalculated based on the standard ethanol content in beers (= 5 %, v/v).

Table 4.9 Concentrations of flavour-active compounds in the final beers derived from different fermentations using ale yeast M2, assessed using headspace GC-MS analysis. Data points represent the mean of triplicate samples \pm standard deviation from independent tests ^a

	<i>Fermentation conditions</i>				
	<i>13P15M</i>	<i>18P15M</i>	<i>18P18M</i>	<i>24P15M</i>	<i>24P24M</i>
<i>Higher alcohols (mg/L)</i>					
<i>Isobutanol</i>	54.67 \pm 2.18	96.73 \pm 3.37	77.88 \pm 5.45	59.40 \pm 3.92	69.96 \pm 3.37
<i>Total amyl alcohol</i>	28.27 \pm 3.77	36.55 \pm 3.53	34.13 \pm 2.07	30.34 \pm 3.61	34.47 \pm 2.69
<i>Total higher alcohols</i>	82.94 \pm 5.92	133.28 \pm 6.86	112.01 \pm 7.45	89.74 \pm 6.26	104.42 \pm 5.65
<i>Esters (mg/L)</i>					
<i>Ethyl acetate</i>	15.88 \pm 0.57	26.37 \pm 2.30	21.54 \pm 3.35	30.20 \pm 3.38	22.15 \pm 3.95
<i>Isoamyl acetate</i>	1.85 \pm 0.36	6.54 \pm 1.63	2.91 \pm 1.85	6.96 \pm 1.24	4.26 \pm 1.89
<i>Ethyl propionate</i>	0.08 \pm 0.00	0.10 \pm 0.09	0.07 \pm 0.02	0.05 \pm 0.03	0.03 \pm 0.03
<i>Ethyl butyrate</i>	0.08 \pm 0.01	0.09 \pm 0.06	0.07 \pm 0.01	0.09 \pm 0.00	0.08 \pm 0.03
<i>Ethyl caproate</i>	0.35 \pm 0.06	0.24 \pm 0.17	0.20 \pm 0.03	0.20 \pm 0.02	0.17 \pm 0.06
<i>Total esters</i>	18.24 \pm 0.81	33.34 \pm 4.22	24.79 \pm 4.85	37.50 \pm 2.94	26.70 \pm 4.86

^a Values of flavour compounds was recalculated based on the standard ethanol content in beers (= 5 %, v/v).

4.2.1.6 The effect of wort gravity and pitching rate on pH

A change in pH can be observed during fermentation, resulting from the formation and excretion of organic acids (such as pyruvate, citrate and acetate) and the release of protons in response to carbohydrate uptake via active transport by yeast cells (Boulton & Quain, 2001). In the current study, the pattern of pH change was monitored by measuring wort and beer samples throughout fermentations (Section 2.7.7).

The initial pH of the commercial wort used was approximately 4.7-5.0 and the pH of the yeast slurry used for inoculation was 3.9-4.0. In all instances, the pH was observed to dramatically decrease between 0-24 hours post-pitching (Figures 4.15-4.16). This immediate reduction in pH was most likely to be influenced by the inherently low pH of the yeast slurry that was added to each fermenter, and the subsequent decrease in pH could be attributed to yeast metabolism as described above, resulting in a minimum pH of approximately 3.6-3.8. The characteristic decrease in pH was followed by a 'recovery' stage, where a slight increase in pH was observed towards the end of fermentation (120 hours post-pitching), resulting in a final mean pH of between 3.8 and 4.1 in each instance, likely to be due to yeast autolysis (Section 1.1.2.8). This was not unexpected as it has been suggested that the final pH is dependent upon the buffering capacity of the wort, initial wort pH and the extent of yeast growth (Heggart, *et al.*, 1999). The only fermentation which showed a slight anomaly was that conducted using strain NCYC1332 under 24P24M conditions. This fermentation showed a particularly rapid decrease in pH (Figure 4.16A), probably due to the faster assimilation of wort sugars resulting from the higher growth levels of yeast observed (Section 4.2.2.1).

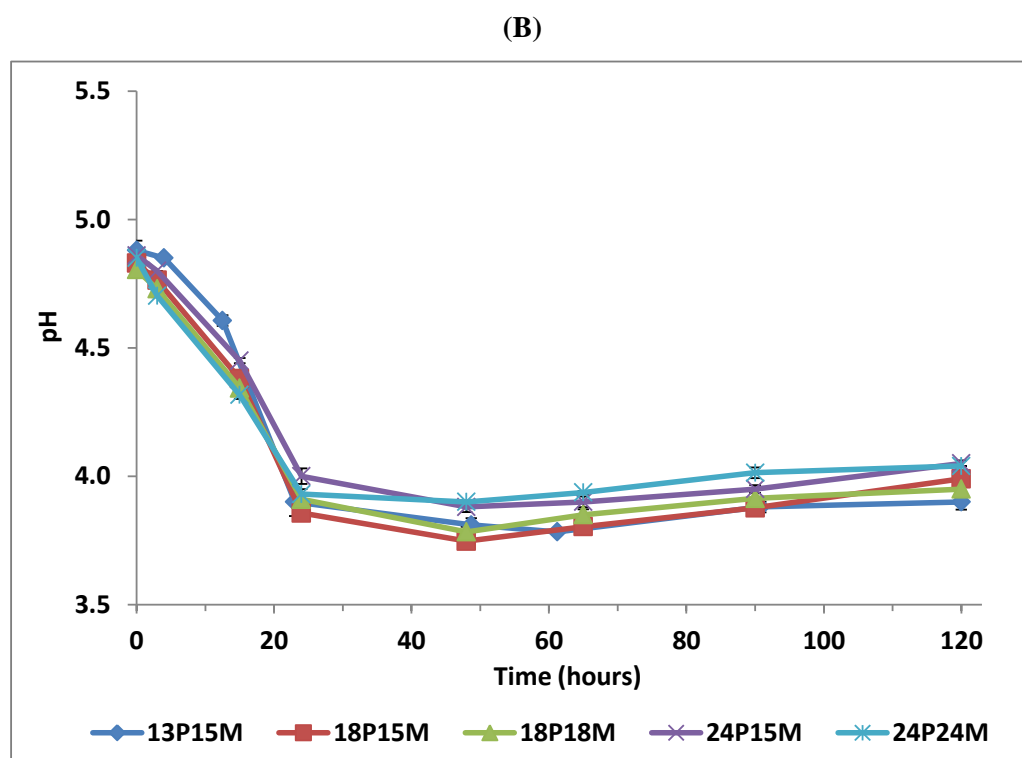
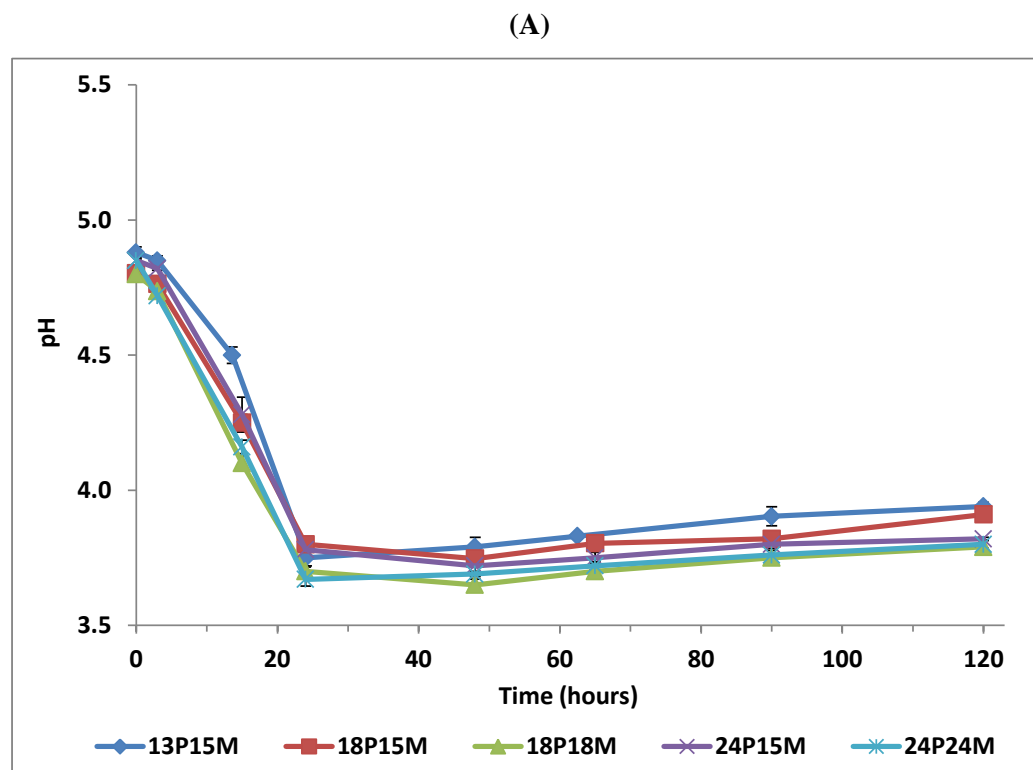


Figure 4.15 Change in pH during fermentations using lager yeast strains Lager1 (A) and W34/70 (B) as a function of time. Samples were taken at approximately 0, 3, 15, 24, 48, 65, 90 and 120 hours and yeast was removed through centrifugation. Data points represent the mean of triplicate samples, with error bars showing the standard deviation.

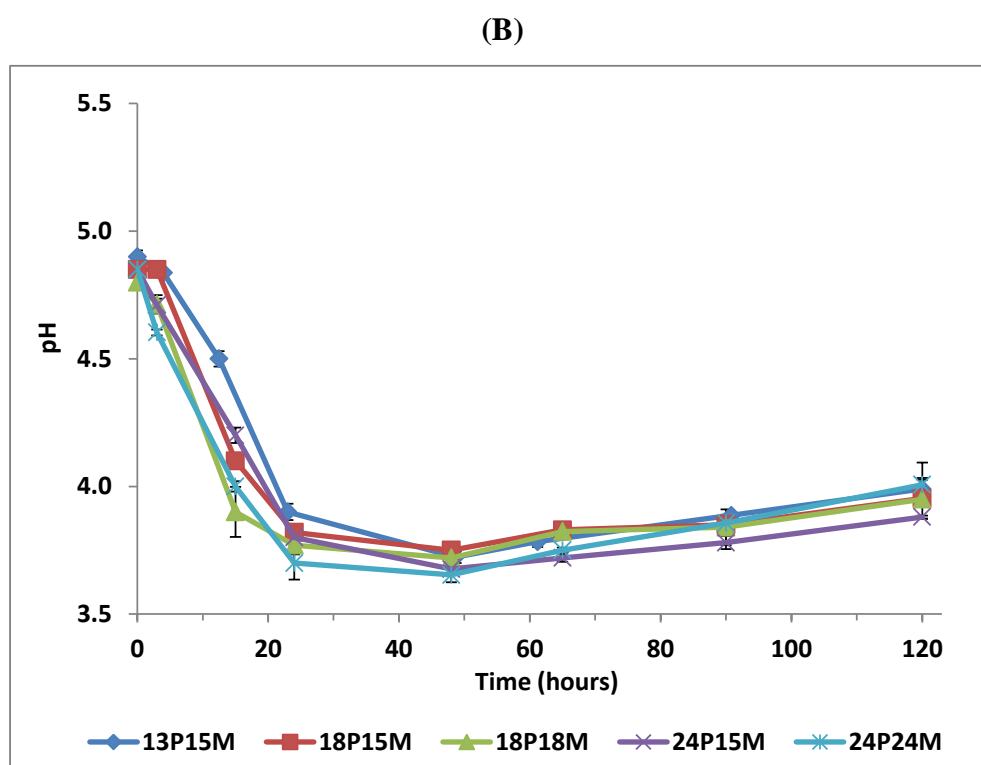
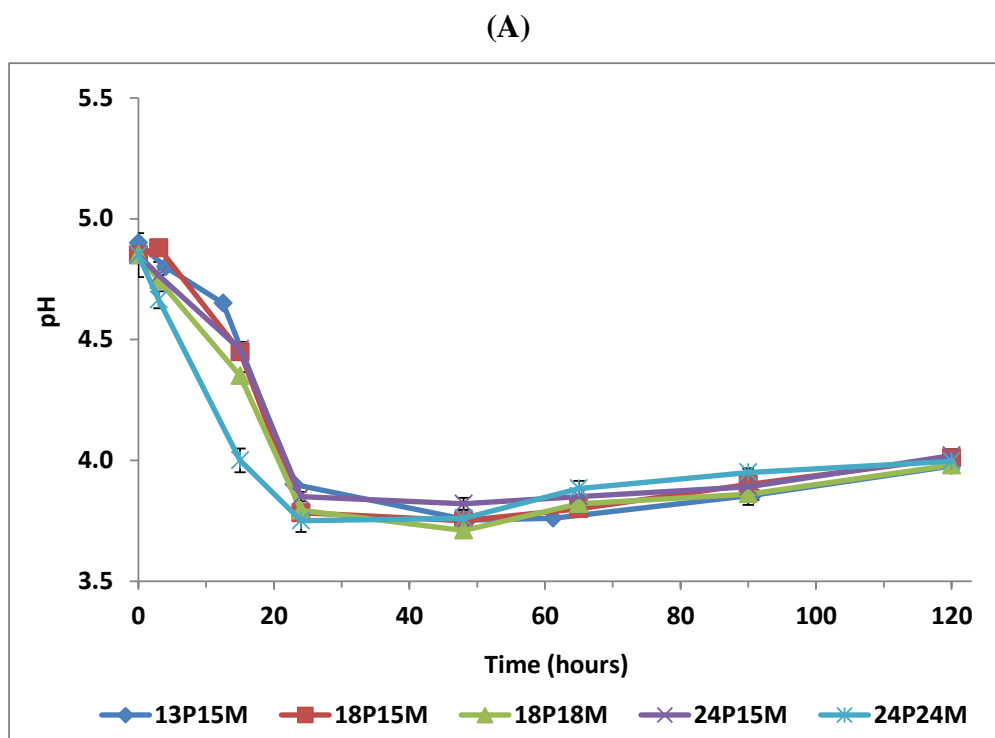


Figure 4.16 Change in pH during fermentations using ale yeast strains NCYC1332 (A) and M2 (B) as a function of time. Samples were taken at approximately 0, 3, 15, 24, 48, 65, 90 and 120 hours and yeast was removed through centrifugation. Data points represent the mean of triplicate samples, with error bars showing the standard deviation.

4.2.2 The impact of wort gravity and pitching rate on yeast cell physiology

4.2.2.1 The effect of wort gravity and pitching rate on cell growth

To determine the impact of wort gravity and pitching rate on population dynamics, fermentations were sampled for cell density and cell viability at regular intervals.

Total cell density at each sample point was determined using a haemocytometer (Section 2.4) and the profiles obtained were used to detail population number during fermentations, characterized by lag, exponential and stationary phase (Figures 4.17-4.18). During the short lag phase, little or no cell growth occurred, however after 4-6 hours cells entered exponential growth. It was observed that the rate of growth was quicker at 24P24M than 24P15M conditions, whereas similar growth pattern was observed between 18P18M and 18P15M conditions, irrespective of yeast strains. For fermentations conducted with a pitching rate of 1.5×10^7 cells/mL, the round of cell division observed (Table 4.10) was increased with the elevated wort gravity for strain Lager1 and NCYC1332, and this was most pronounced for ale strain NCYC1332 (Figure 4.18A). In addition, when comparing 18P15M to 18P18M and 24P15M to 24P24M conditions, it was observed that the round of cell division decreased at a higher pitching rate (18P18M and 24P24M) in all the four strains studied (Table 4.10). However, it was interesting to notice that the lager strain W34/70 displayed a comparably lower amount of growth, and that for this strain the maximum growth was similar under all conditions except for 24P24M condition (Figure 4.17B). After the deceleration phase, the yeast cells entered stationary phase of the population growth curve, and cell number was observed to remain relatively constant until the end of fermentation in each scenario. This was true for all of the yeast strains analysed, except for NCYC1332, which showed decreased cell numbers with the advance of stationary phase, especially pronounced during 24 °P fermentations (Figure 4.18A).

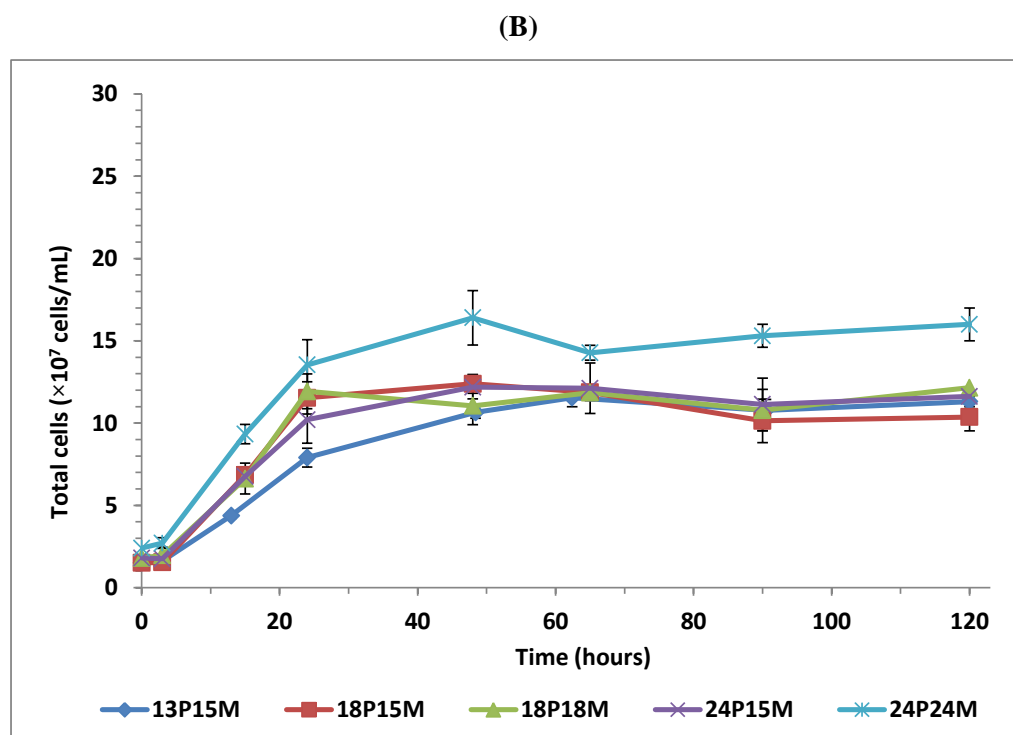
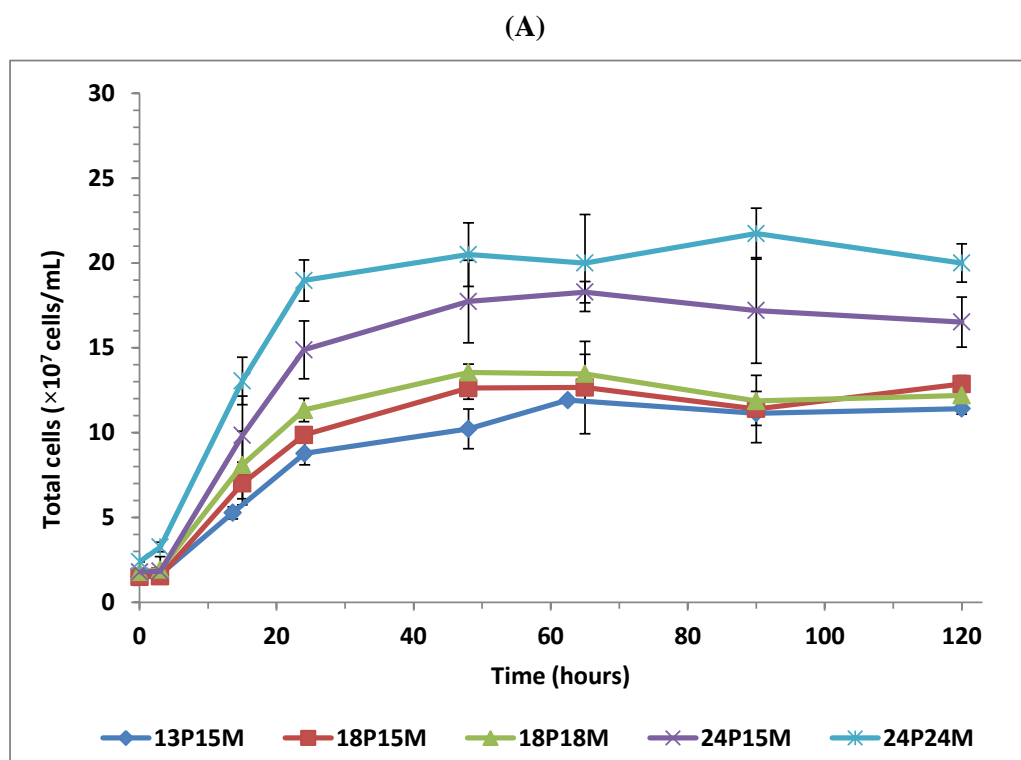


Figure 4.17 Mean cell numbers present in fermentations using lager yeast strains Lager1 (A) and W34/70 (B) as a function of time. Samples were taken at approximately 0, 3, 15, 24, 48, 65, 90 and 120 hours and the number of suspended cells was assessed using haemocytometer. Data points represent the mean of triplicate samples, with error bars showing the standard deviation.

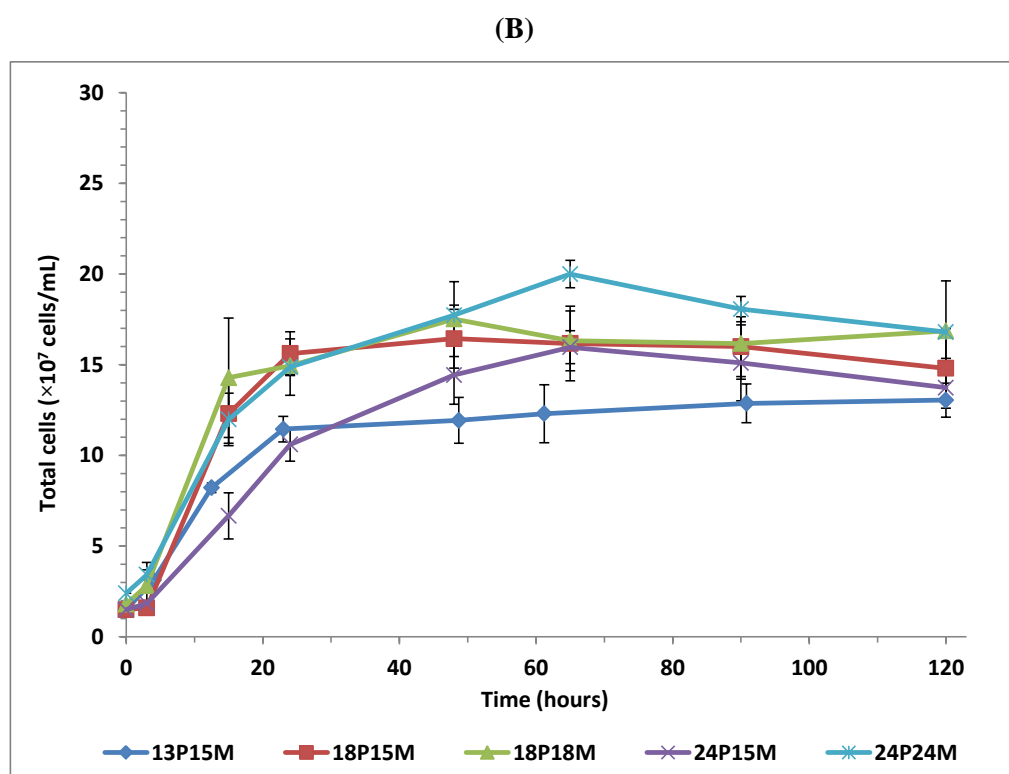
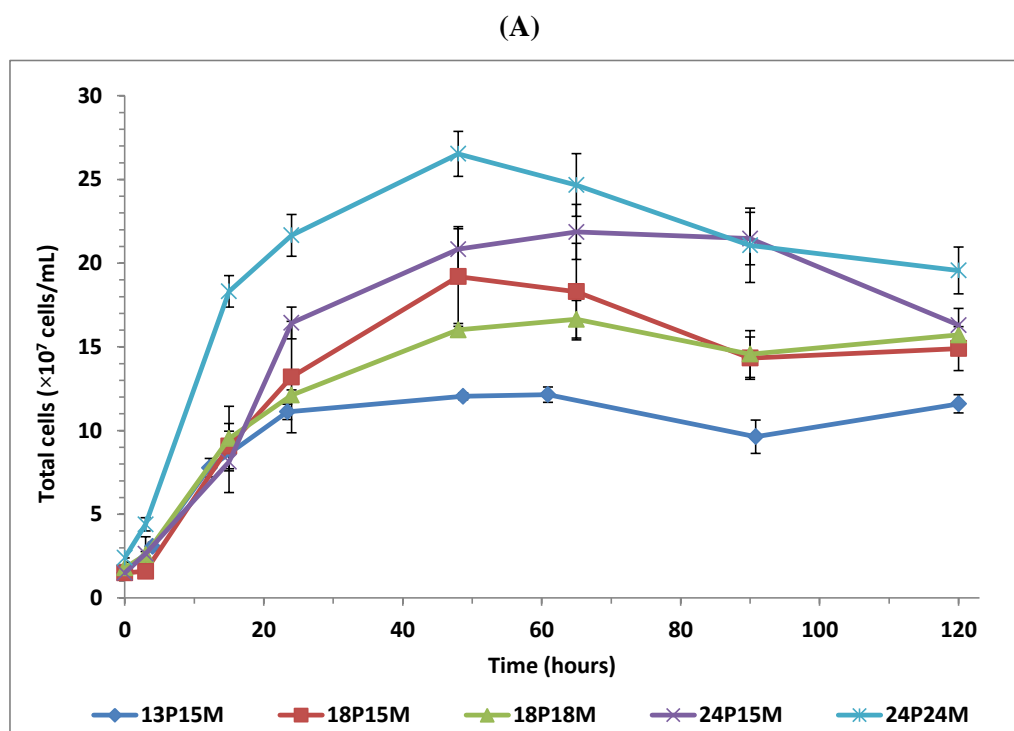


Figure 4.18 Mean cell numbers present in fermentations using ale yeast strains NCYC1332 (A) and M2 (B) as a function of time. Samples were taken at approximately 0, 3, 15, 24, 48, 65, 90 and 120 hours and the number of suspended cells was assessed using haemocytometer. Data points represent the mean of triplicate samples, with error bars showing the standard deviation.

Table 4.10 Round of cell division under different fermentation conditions. Data represent the round of cell division from the initial cell density to the maximum cell density.

<i>Yeast strain</i>	<i>Round of cell division</i>				
	<i>13P15M</i>	<i>18P15M</i>	<i>18P18M</i>	<i>24P15M</i>	<i>24P24M</i>
Lager1	3.0	3.1	2.8	3.5	3.1
W34/70	2.9	3.0	2.7	3.0	2.7
NCYC1332	3.0	3.2	3.1	3.8	3.4
M2	3.1	3.4	3.2	3.3	3.0

4.2.2.2 The effect of wort gravity and pitching rate on cell viability

The viability of yeast in suspension was monitored during each set of fermentations using the methylene blue staining technique (Section 2.4.1).

In each of the five conditions studied, the viability of yeast was observed to decrease during the course of fermentation (Figures 4.19-4.20). For fermentations conducted with a pitching rate of 1.5×10^7 cells/mL, the viability at high gravities (18 °P and 24 °P) decreased faster than that at standard gravity (13 °P) and the cropped viability was decreased with the elevated starting gravity, irrespective of brewing classification (lager or ale). Also, an accelerated drop in viability was observed at higher pitching rates, when comparing 18P18M and 24P24M to 18P15M and 24P15M, respectively. This was true for all the strains except for W34/70, which displayed similar pattern of viability loss at both 18 °P and 24 °P fermentations. Despite this it should be noted that initial cell numbers did not appear to influence the

cropped viability; there was no significant difference ($P < 0.05$) in the viability loss between the two pitching rates at the end of fermentations (18 °P or 24 °P) for any of the yeast strains examined (Figures 4.19-4.20). Moreover, lager strains displayed a more moderate decrease in viability than the ale strains used in this study. To demonstrate this, it can be seen that at high gravities cropped yeast from fermentations conducted using the lager strain W34/70 (Figure 4.19B) and the ale strain NCYC1332 (Figure 4.20A) showed the greatest and lowest viabilities, respectively.

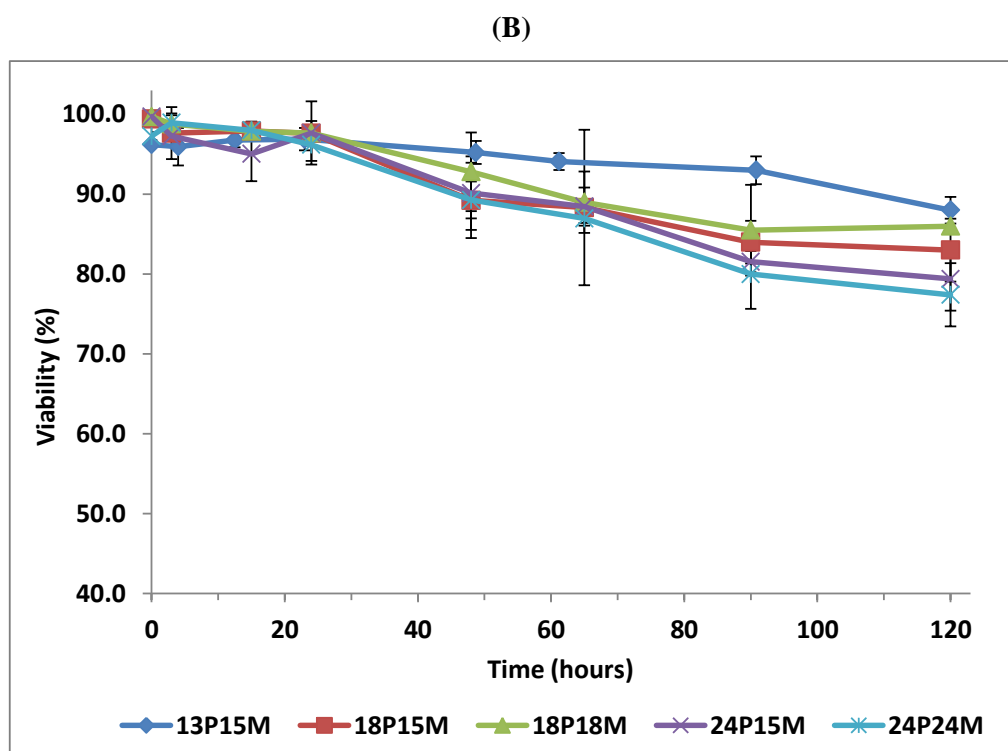
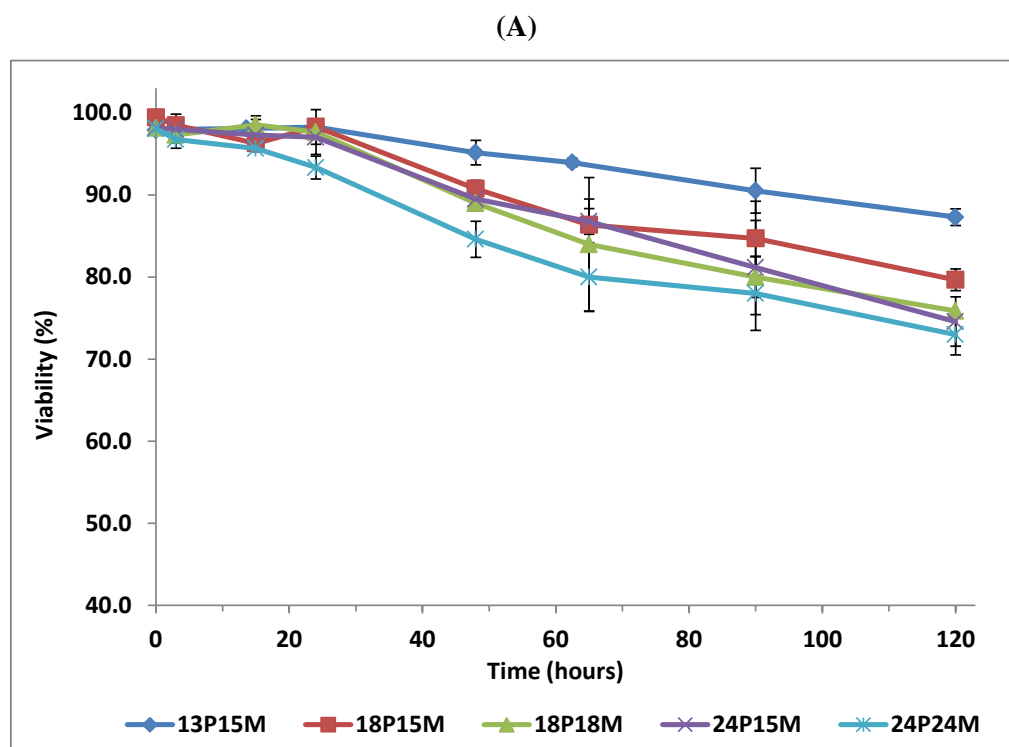


Figure 4.19 Percentage of viable cells during fermentations using lager yeast strains Lager1 (A) and W34/70 (B) as a function of time. Samples were taken at approximately 0, 3, 15, 24, 48, 65, 90 and 120 hours and viability was determined using methylene blue staining. Data points represent the mean of triplicate samples, with error bars showing the standard deviation.

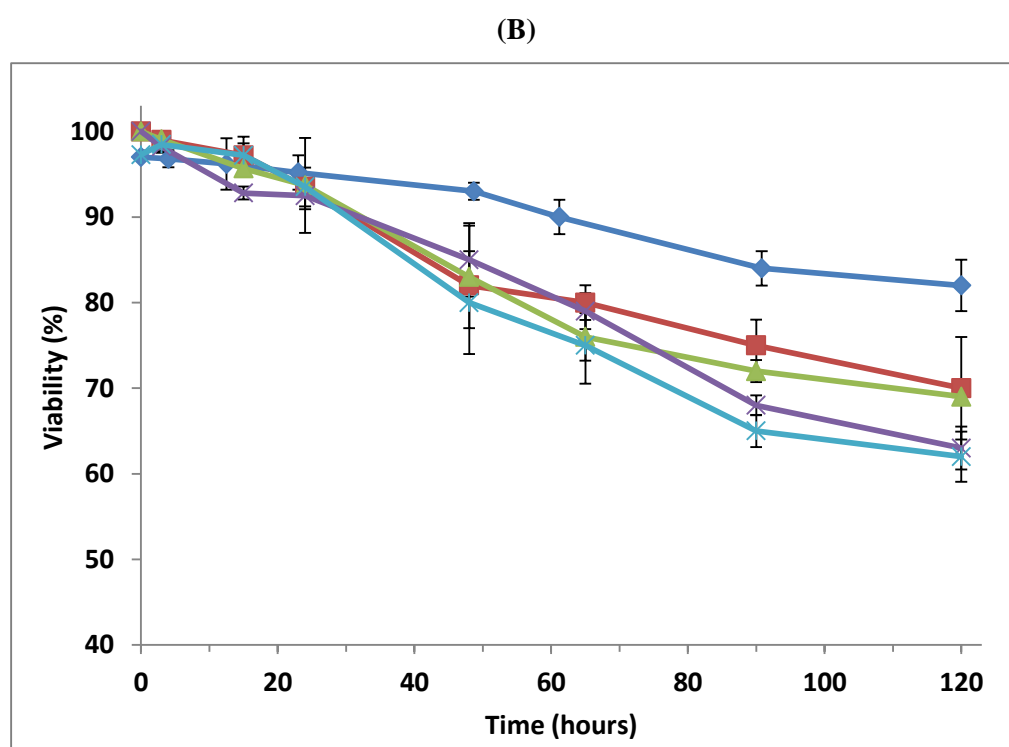
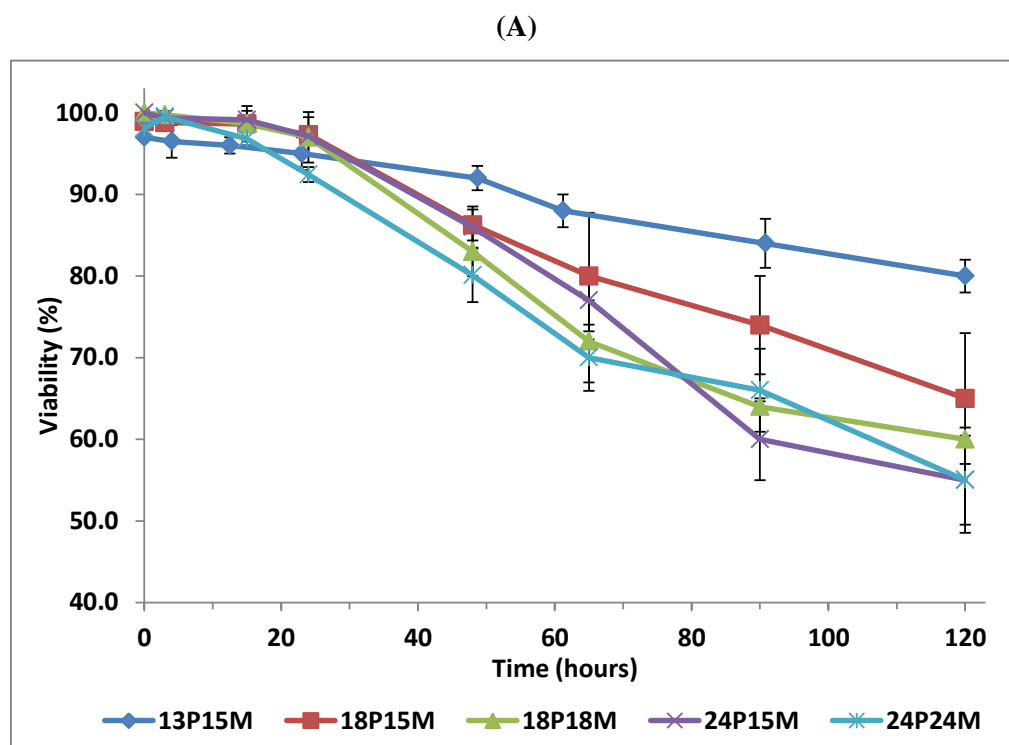


Figure 4.20 Percentage of viable cells during fermentations using ale yeast strains NCYC1332 (A) and M2 (B) as a function of time. Samples were taken at approximately 0, 3, 15, 24, 48, 65, 90 and 120 hours and viability was determined using methylene blue staining. Data points represent the mean of triplicate samples, with error bars showing the standard deviation.

4.2.2.3 The effect of wort gravity and pitching rate on yeast trehalose content

In addition to an energetic role, trehalose is believed to play an important function in stress tolerance and is produced in response to a variety of stress factors. Since trehalose is a disaccharide composed of two units of glucose, in this study it was analysed by enzymatically cleaving the trehalose molecule into its components followed by quantification of glucose using a commercial assay kit. The resultant concentration of glucose therefore reflects the total intracellular trehalose present in a defined number of cells (Section 2.8.1).

Low levels of trehalose were observed during lag phase of all fermentations (Figures 4.21-4.22), most likely resulting from glucose repression and inactivation of trehalose biosynthesis pathways (Bell, *et al.*, 1992, Devirgilio, *et al.*, 1993, Vuorio, *et al.*, 1993, Boulton & Quain, 2001). Furthermore, it can be seen from that in all instances trehalose was initially degraded, but thereafter was synthesized rapidly between 20-60 hours, after which the presence of trehalose was observed to gradually decrease towards the end of fermentation. When comparing brewing yeast types, it can be seen that wort gravity and pitching rate had an effect on accumulation of trehalose in lager strains (Figure 4.21), but that this was not evident in ale strains (Figure 4.22). For lager strains, a combination of high pitching rate and gravity (24P24M) led to the highest amount of trehalose being produced whereas when using 18 °P wort, pitching rate had similar impact on trehalose production (Figure 4.21). Conversely to the above, lager fermentations conducted using 13 °P wort showed the lowest amount of trehalose production. The pattern of trehalose production observed for lager strains was not seen in either of the ale strains examined (Figure 4.22). For both NCYC1332 and M2, the maximum trehalose value observed was not significantly affected by pitching rate or starting gravity. In addition, ale strains appeared to produce less trehalose in general, with strain M2 producing particularly low levels of this disaccharide irrespective of fermentation conditions.

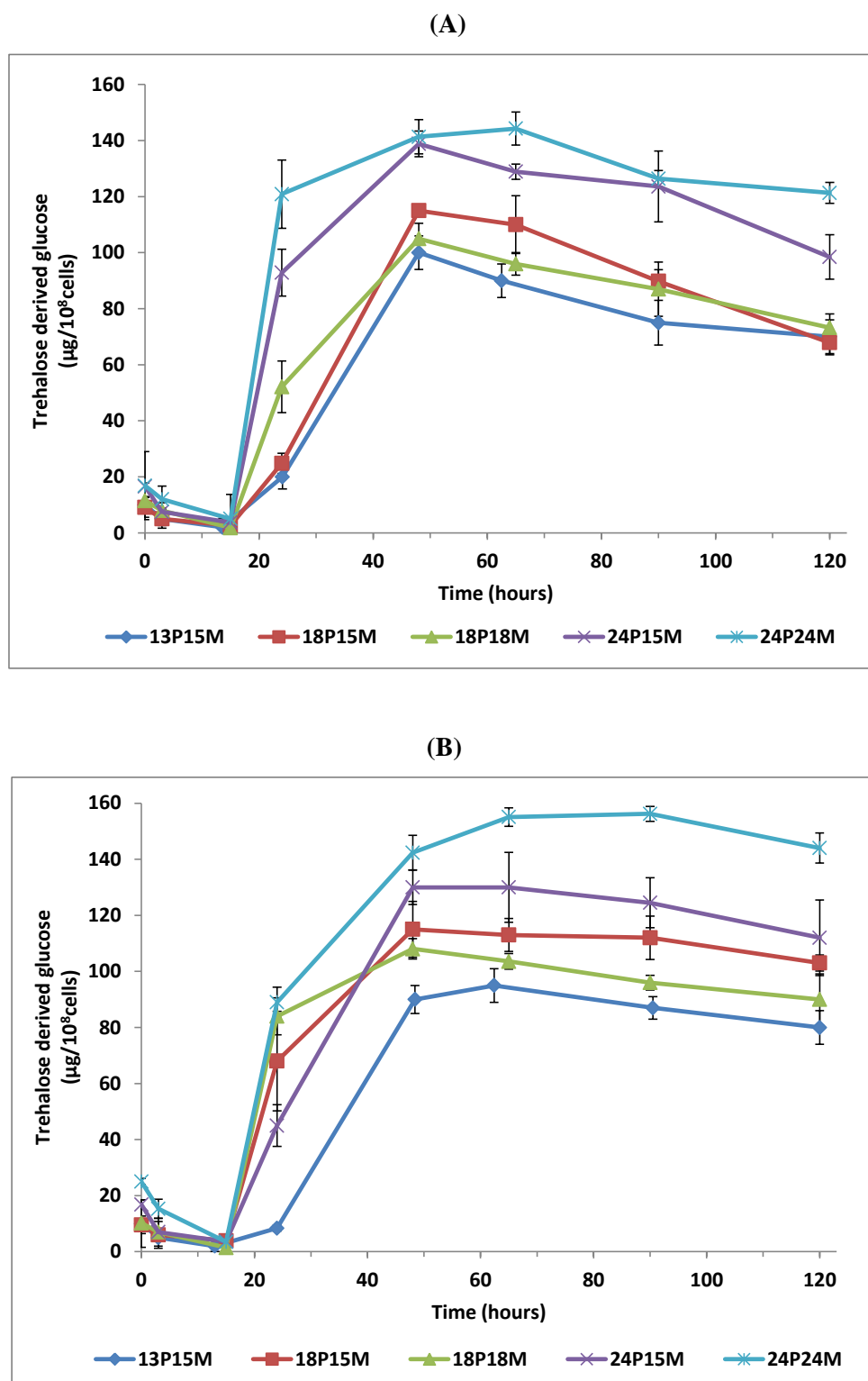


Figure 4.21 Concentrations of trehalose during fermentations with lager strains Lager1 (A) and W34/70 (B) as a function of time. Samples were taken at approximately 0, 3, 15, 24, 48, 65, 90 and 120 hours, and yeast cells were collected through centrifugation. Trehalose was enzymatically digested and the resultant glucose was assessed using a commercial assay. Data points represent the mean of triplicate samples, with error bars showing the standard deviation.

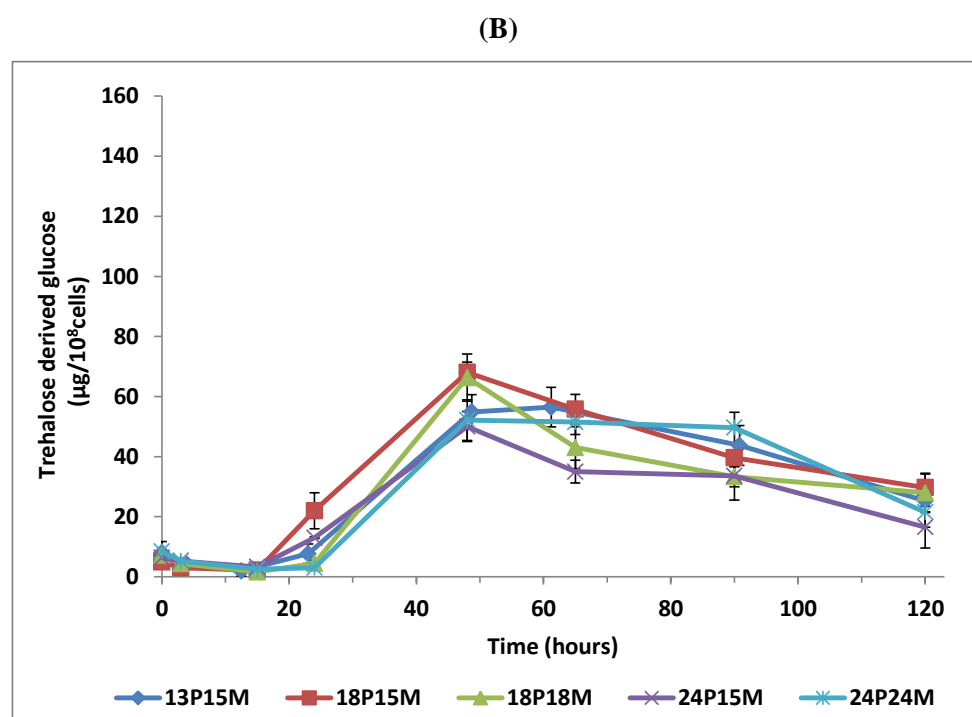
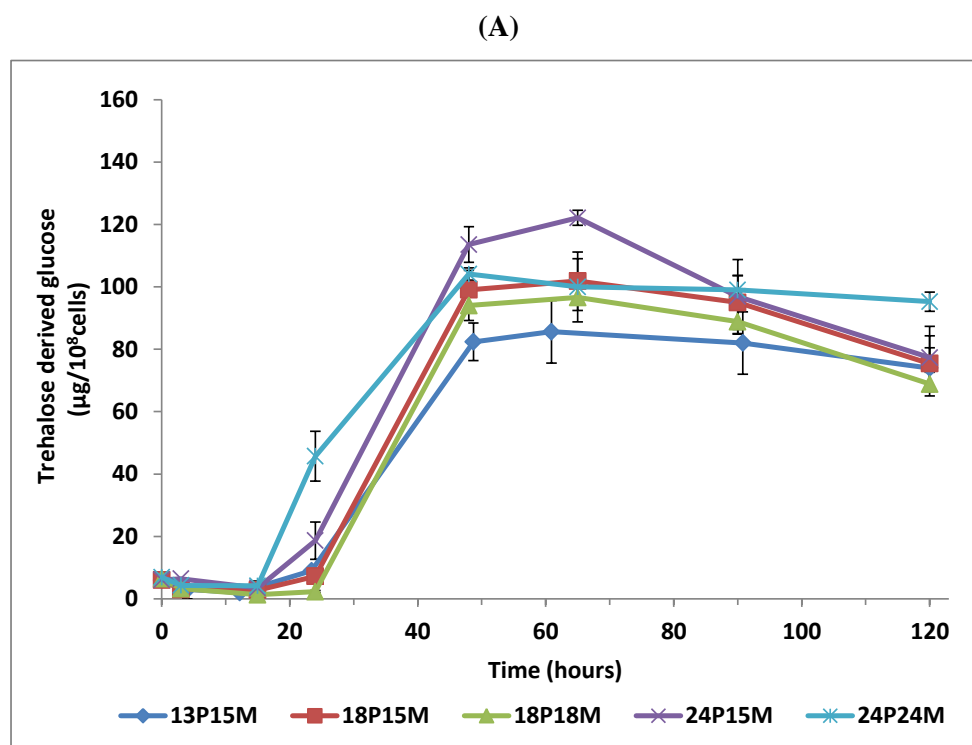


Figure 4.22 Concentrations of trehalose during fermentations using ale strains NCYC1332 (A) and M2 (B) as a function of time. Samples were taken at approximately 0, 3, 15, 24, 48, 65, 90 and 120 hours, and yeast cells were collected through centrifugation. Trehalose was enzymatically digested and the resultant glucose was assessed using a commercial assay. Data points represent the mean of triplicate samples, with error bars showing the standard deviation.

4.2.2.4 The effect of wort gravity and pitching rate on glycogen content

Glycogen is a multi-branched glucose polymer (Torija, *et al.*, 2005) which is generally considered to be a major storage carbohydrate in yeast, providing a readily available source of energy for the organism (Francois & Parrou, 2001). Similar to trehalose determination, cellular glycogen was enzymatically digested and the resultant glucose was assayed to reflect the levels of glycogen in a defined number of yeast cells (Section 2.8.1).

Profiles of intracellular glycogen throughout lager and ale fermentations are depicted in Figure 4.23 and 4.24, respectively. In the majority of instances, accumulation of glycogen peaked between approximately 20 and 40 hours post-pitching, and the values typically remained stable thereafter until the end of the fermentation. However, at the lowest gravity, this trend was not observed, with all fermentations conducted at 13 °P demonstrating a reduction in glycogen after the initial peak, irrespective of pitching rate or strain type. It was also interesting to note that fermentations conducted using higher pitching rates (18P18M and 24P24M) exhibited lower maximum levels of glycogen than when lower pitching rates were employed (18P15M and 24P15M). This was true for each strain except for M2, which showed similar glycogen concentrations at 24P15M and 24P24M conditions (Figure 4.24B). Although higher levels of glycogen were accumulated when a lower pitching rate was applied, this was not always reflected in the rate of accumulation. For example, at 24 °P glycogen accumulated faster (earlier) when a higher pitching rate (24P24M) was applied. In contrast, during 18 °P fermentations, glycogen accumulation began the quickest (earliest) at the lower pitching rate (18P15M) for all strains except for Lager1 (Figure 4.23A).

When comparing standard gravity (13 °P) fermentations to other gravities, it can be seen that 13P15M conditions displayed similar peak profiles to 18P15M conditions. Furthermore, when yeast derived from 13P15M fermentations was cropped and the glycogen content was

measured, no significant difference ($P < 0.05$) was found between 13P15M, 18P18M and 24P24M conditions, irrespective of the yeast strain applied. This supports the observation above suggesting that higher pitching rates may have a detrimental impact on yeast glycogen levels in HG and VHG fermentations. It should be noted that, as previously (Section 4.2.2.3), the ale strain M2 showed slight physiological differences to the other strains. Although analysis of fermentations with low pitching rates indicated that higher glycogen levels were typically achieved, this was not observed for this particular ale strain, with no significant difference in glycogen at 24 °P fermentations, irrespective of pitching rate (Figure 4.24B).

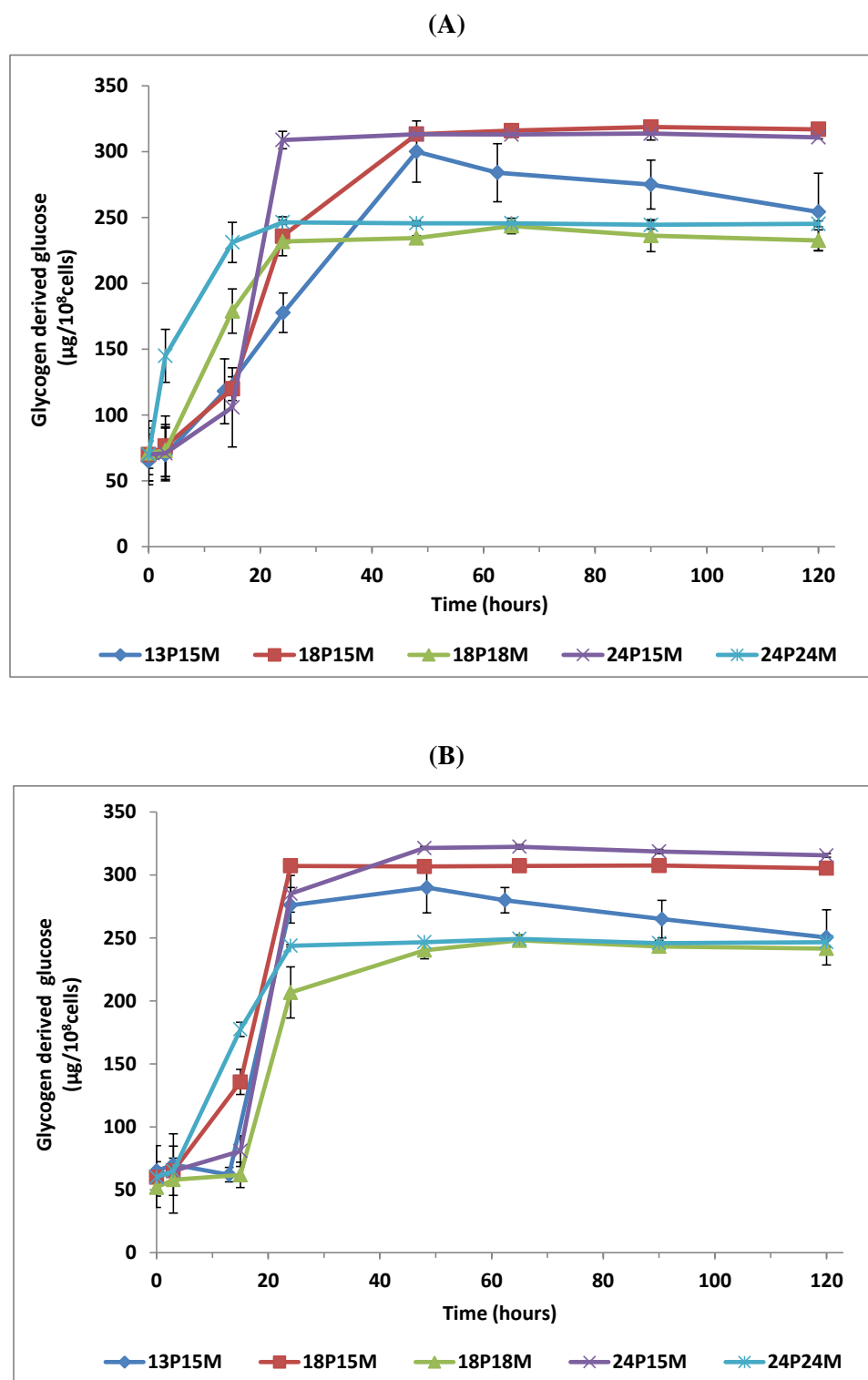


Figure 4.23 Concentrations of glycogen during fermentations using lager strains Lager1 (A) and W34/70 (B) as a function of time. Samples were taken at approximately 0, 3, 15, 24, 48, 65, 90 and 120 hours and yeast cells were collected through centrifugation. Glycogen was enzymatically digested and the resultant glucose was assessed using a commercial assay. Data points represent the mean of triplicate samples, with error bars showing the standard deviation.

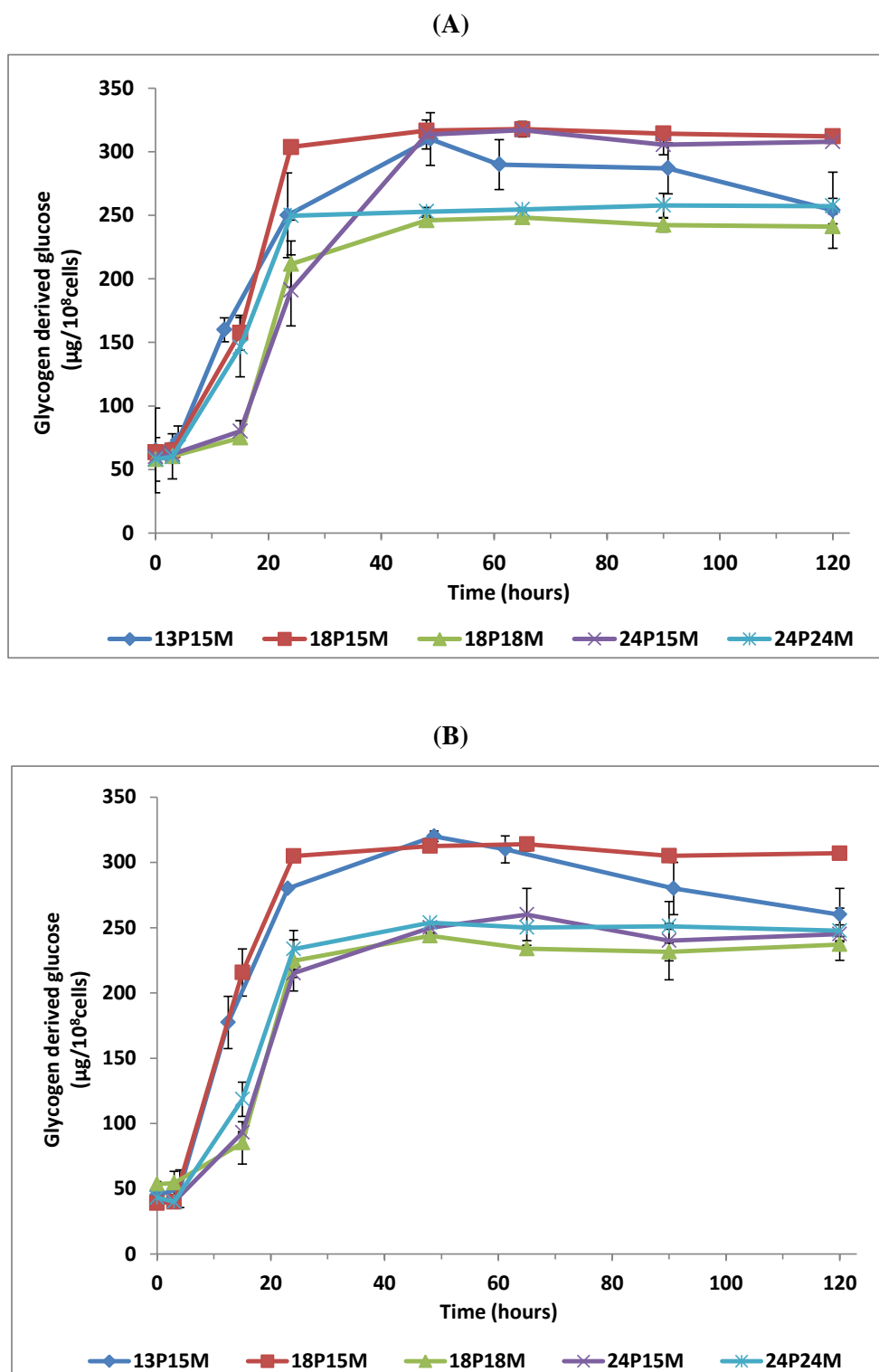


Figure 4.24 Concentrations of glycogen during fermentations using ale strains NCYC1332 (A) and M2 (B) as a function of time. Samples were taken at approximately 0, 3, 15, 24, 48, 65, 90 and 120 hours and yeast cells were collected through centrifugation. Glycogen was enzymatically digested and the resultant glucose was assessed using a commercial assay. Data points represent the mean of triplicate samples, with error bars showing the standard deviation.

4.2.2.5 The effect of wort gravity and pitching rate on glycerol production

Glycerol is produced to maintain cytosolic redox balance during fermentation, as well as to act as a compatible solute during osmoregulation in yeast (Hohmann, 2002). In the current study, in order to determine the impact of wort gravity and pitching rate on its biosynthesis, the glycerol content of wort and beer samples was determined using HPLC and expressed in g/L (Section 2.7.3).

There was no glycerol in the original wort and the concentration of glycerol was observed to increase at the beginning of both lager and ale fermentations, although the rate of production was strain-dependent (Figures 4.25-4.26). The point of maximum accumulation was typically achieved at 50-60 hours post-pitching and the values remained constant thereafter until the end of fermentations. Fermentations conducted under 24P24M and 13P15M conditions showing the highest and lowest glycerol concentrations, respectively, regardless of the yeast strain used. For fermentations with pitching rate of 1.5×10^7 viable cells/mL, the four yeast strains showed an increase in glycerol according to original wort gravity and this was most pronounced in strain Lager1. In addition, Lager1 produced a significantly higher ($P < 0.05$) amount of glycerol at 18P18M and 24P24M compared to 18P15M and 24P15M, respectively (Figure 4.25A). For the other yeast strains (Figure 4.25B and Figure 4.26), when comparing pitching rates at 18 °P (18P15M compared to 18P18M), it can be seen that there was slight difference in glycerol formation at the end of fermentation. However, there was a markedly difference in glycerol production between pitching rates at 24 °P (24P15M and 24P24M) and this was most pronounced in strains Lager 1 (Figure 4.25A) and NCYC1332 (Figure 4.26A).

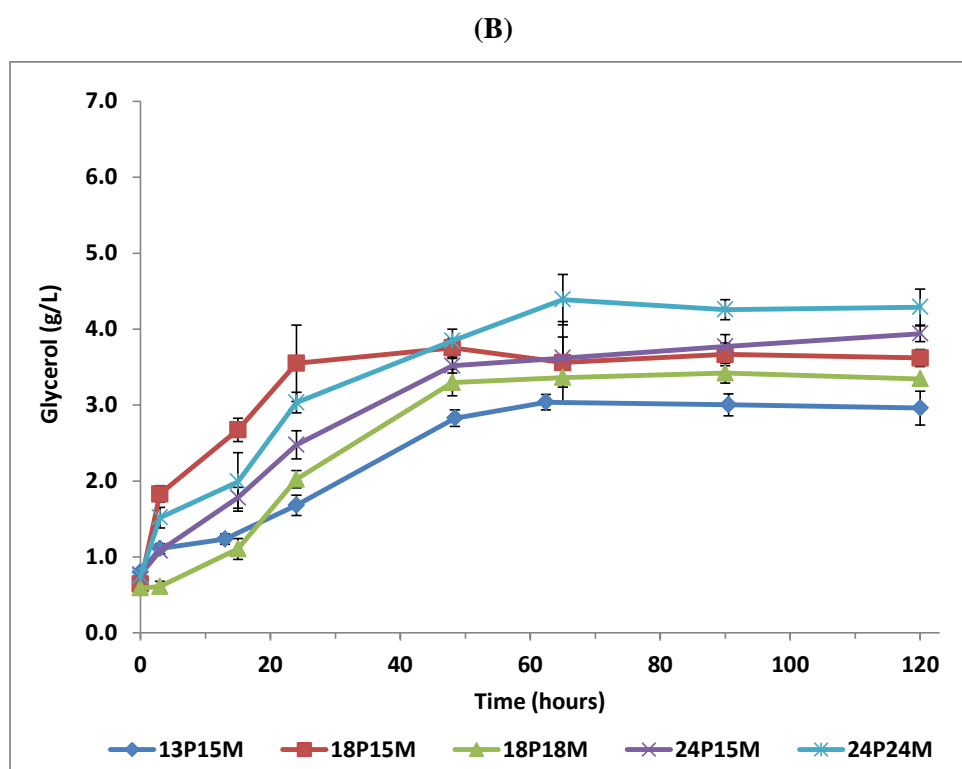
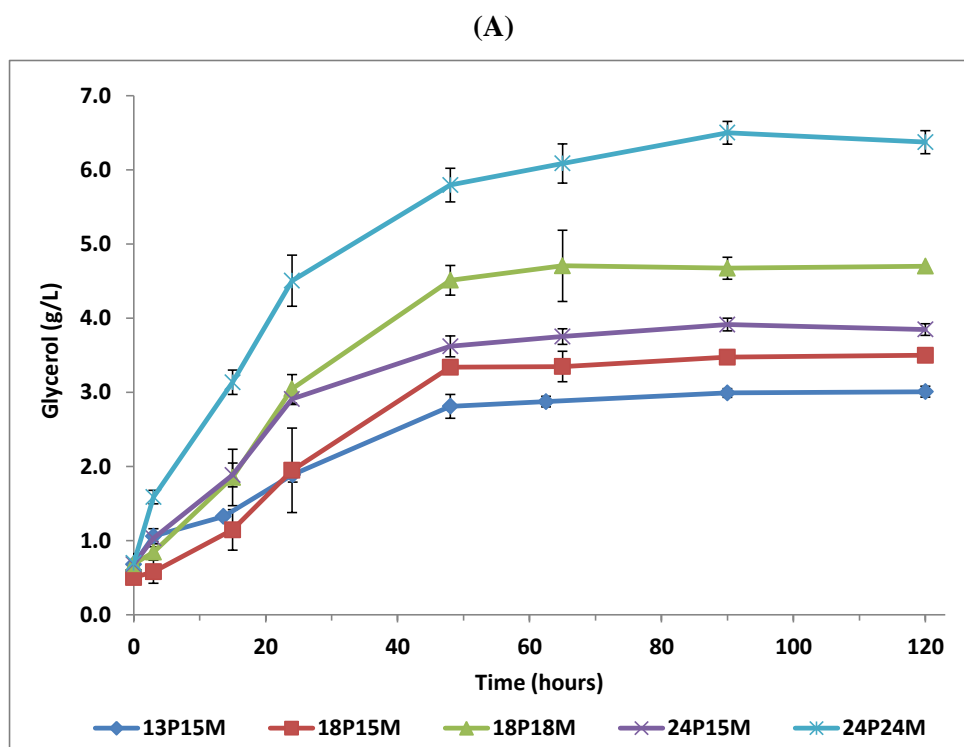


Figure 4.25 Concentrations of glycerol during fermentations using lager strains Lager1 (A) and W34/70 (B) as a function of time, assessed by HPLC. Samples were taken at approximately 0, 3, 15, 24, 48, 65, 90 and 120 hours, and yeast was removed through centrifugation. Data points represent the mean of triplicate samples, with error bars showing the standard deviation.

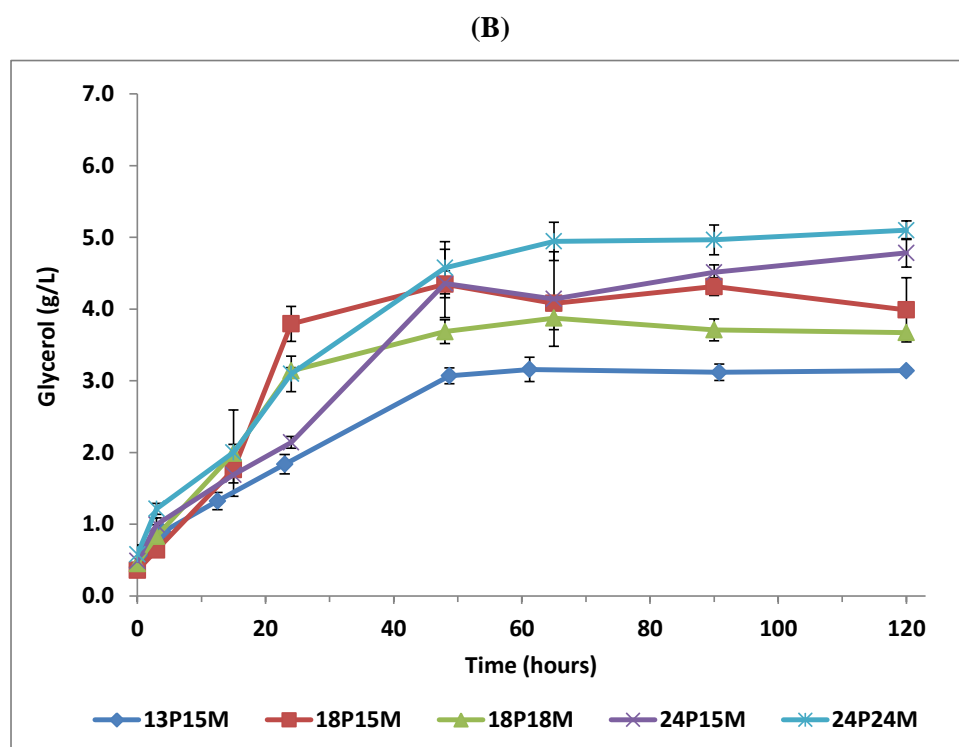
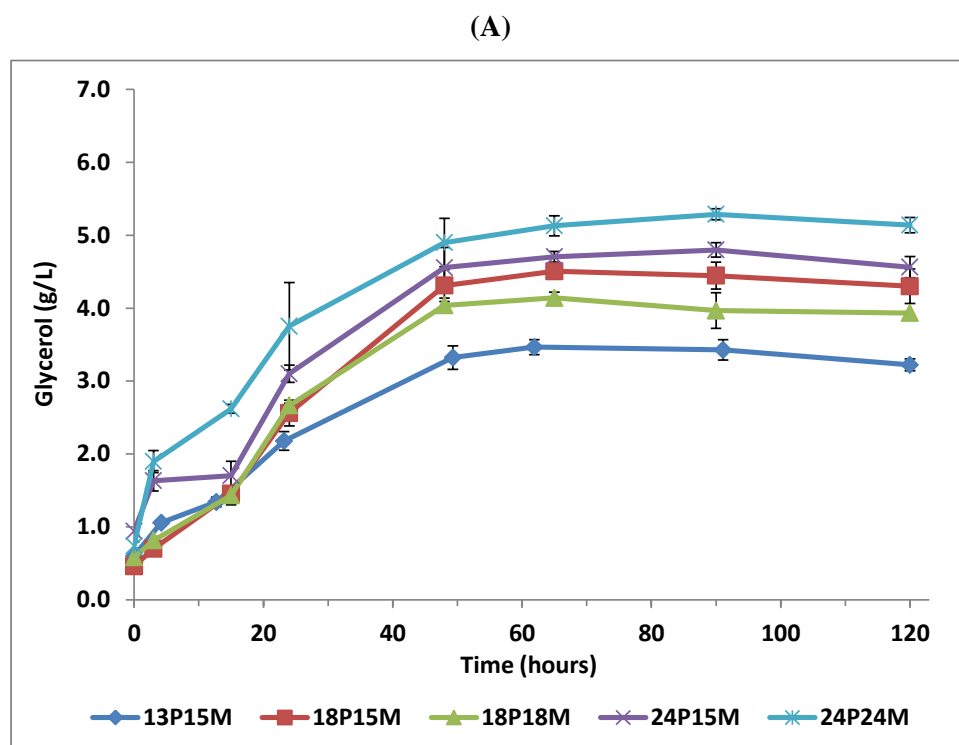


Figure 4.26 Concentrations of glycerol during fermentations using ale strains NCYC1332 (A) and M2 (B) as a function of time, assessed by HPLC. Samples were taken at approximately 0, 3, 15, 24, 48, 65, 90 and 120 hours, and yeast was removed through centrifugation. Data points represent the mean of triplicate samples, with error bars showing the standard deviation.

4.3 Discussion

In the present study, yeast physiological activity and key performance indicators were determined during a series of lab-scale fermentations using lager and ales strains in conjunction with 13 °P, 18 °P and 24 °P worts. For each set of fermentations pitching rate and oxygen condition were also adjusted, based on standard practice, to examine their combined effects on yeast fermentation and carbon products.

4.3.1 The impact of wort gravity and pitching rate on fermentation performance

As expected, it was observed that wort carbohydrates were utilized in a sequential manner, as a consequence of carbon catabolite repression (Boulton & Quain, 2001, Gibson, *et al.*, 2008). However, in general, fermentations conducted using higher initial wort gravities resulted in a less complete conversion of sugar, of which maltotriose was the most evident (Figures 4.9-4.10). This is a significant issue since the presence of unfermented sugar in beer produced using high gravity worts represents an economic loss for brewers, resulting in lower ethanol yield from wort carbohydrate and possibly also affecting organoleptic characteristics (Vidgren, *et al.*, 2009). However, poor maltotriose utilisation is a well characterised artefact of high gravity brewing and supports previous studies which have demonstrated this effect (Zheng, *et al.*, 1994, Vidgren, *et al.*, 2009, Yu, *et al.*, 2012). It is known that the influx of more readily assimilable sugars into the glycolysis pathway can act to repress fermentation of maltotriose. Interestingly this issue has been shown to be reversible by manipulation of functional maltotriose transporter genes such as *AGT1* (Han, *et al.*, 1995). Overexpression of the *AGT1* gene significantly enhances maltotriose fermentation (Stambuk, *et al.*, 2006) and it has been shown that transformants exhibit a faster fermentation rate at VHG (24 °P) wort, leading to beers containing more ethanol and less residual maltotriose (Vidgren, *et al.*, 2009). Although genetically modified organism (GMO) are currently prohibited due to little or no

customer tolerance, in the future there is potential to establish appropriate screening systems, in conjunction with more natural approaches, to develop strains with efficient maltotriose fermentation capacity under industrial brewing conditions. Moreover, differences of *AGT1* between ale and lager yeast have been explored by gene sequencing. Vidgren, *et al.* (2005) reported that *AGT1* genes from two apparently unrelated lager strains contain a premature stop codon and do not encode functional transporters, whereas those of the other two ale yeast strains encode functional transporters. This premature stop codon of *AGT1* genes was later observed in all eight of the lager strains but was not in any of the four ale strains tested (Vidgren, *et al.*, 2007). Furthermore, a novel kind of α -glucoside transporter encoded by *MTT1* (also called *MTY1*) gene was reported (Dietvorst, *et al.*, 2005, Salema-Oom, *et al.*, 2005). The Mtt1 transporters have high activity toward maltotriose than maltose (Salema-Oom, *et al.*, 2005), and Vidgren, *et al.* (2007) found that *MTT1* genes were present in all of the lager strains tested but in none of the ale strains tested.

Aside from metabolic repression due to the sugar source, it has been shown that increasing wort gravity may also have an indirect influence on carbohydrate uptake (Takahashi, *et al.*, 1997). For example, increasing initial wort osmotic pressure by adding sorbitol (an unfermentable sugar for brewing yeast) or sugar adjuncts has been shown to inhibit maltotriose utilization (Zheng, *et al.*, 1994, Lei, *et al.*, 2012). Yu, *et al.* (2012) suggested that this phenomenon may be a result of increased energy charge and decreased α -glucoside transport rate, as well as reduced glycolytic enzyme activity. The same authors proposed that this may in turn be due to strong metabolic repression and inhibition reactions caused by environmental stress factors. Finally, it should also be noted that it has been suggested that the structural complexity of the nitrogen source, such as the complex mixture of amino acids, peptides, proteins, vitamins, ions, nucleic acids in brewer's wort (Stewart & Russell, 1998), could influence the capacity of different strains to assimilate sugars during fermentation

(Batistote, *et al.*, 2006, Lei, *et al.*, 2012). While this was not examined in detail in the current study, it is certainly an area of research which is worth examining in the future.

Ethanol is the major product derived from yeast central carbon metabolism and is an important key performance indicator for HG and VHG brewing. In the present study, ethanol production increased proportionally to the initial wort gravity, as expected. In particular, VHG in combination with high pitching rate (24P24M) resulted in a more complete conversion of wort carbohydrate (particularly maltotriose) to ethanol. The effect of cell density on ethanol yield has been studied previously, although contradictory results have been reported. Erten, *et al.* (2007) conducted fermentations pitched at levels of 2.5, 5.0, 7.5 and 10.0×10^7 viable cells/mL and ethanol formation was apparently unaffected by the pitching rate. In contrast, O'Connor Cox and Ingledew (1991) observed higher ethanol concentrations when a higher pitching rate (8×10^7 cells/mL) was used when compared to a lower pitching rate (1.6×10^7 cells/mL). During the course of high gravity (16 °P) fermentations in this instance, ethanol values increased from 6.13 to 7.63 % (v/v). This is largely expected to be a result of an energy trade off within yeast central carbon metabolism, which will be investigated further in Chapter 5.

Formation of carbon dioxide was shown to be stoichiometric to ethanol production and carbohydrate utilisation during fermentation in this study. This supports the previous work of Daoud and Searle (1990) who studied patterns of CO₂ evolution using laboratory and pilot scale fermentations. At laboratory scale these authors demonstrated a correlation coefficient of 0.9944 between CO₂ evolved and ethanol yield, and 0.99 between CO₂ evolved and carbohydrates utilised. It should be noted, however, that at brewery scale (100 hl fermentations), gas evolution would not be observed until wort became saturated, a potential source of inconsistency. Stassi, *et al.* (1987) and Stassi, *et al.* (1991) found a correlation

between CO₂ formation and a decline in gravity using thermal mass flow meters to measure CO₂ evolution rates both at laboratory and production scale brewing fermentations. In addition, the authors demonstrated a high correlation between CO₂ evolution and the generation of ethanol, the extent of yeast growth, the decline in wort pH and the content of dissolved sulphur dioxide. Therefore it is evident that profiles of CO₂ evolution can be used effectively to monitor fermentation progress (Quain, *et al.*, 1985, Powell, *et al.*, 2003). The major problems in this technique lie in the lack of data which can be obtained during early fermentation, where little or no CO₂ formation occurs since the critical processes of oxygen assimilation and yeast sterol synthesis take place (Annemüller & Manger, 1997, Boulton & Quain, 2001). However, despite these potential issues, the data generated in this study indicates that at small scale fermentation with proper agitation, CO₂ evolution can be accurately monitored and correlates well to ethanol production.

Regarding the flavour profile of final beers, it should be noted that this was performed simply to obtain an indication of any anomalies arising due to fermentation conditions and was not the primary focus of this work. As noted previously, 18 °P and 24 °P brews were conducted using un-hopped wort while 13 °P fermentations were conducted using hopped wort. Whilst this is unlikely to have impacted on the performance of the yeast strain, an accurate evaluation of differences in flavour characteristics is difficult to achieve. In the current study, the impact of pitching rate on ester formation appeared to be minor, irrespective of yeast strain. This result was in agreement with the findings of Erten, *et al.* (2007), who reported that the pitching rate had no clear effect on the ester concentrations. Moreover, the data indicated that the increased wort gravity had a positive effect on the production of ethyl acetate, consistent with previous observations of Piddocke, *et al.* (2009). These authors also reported that the concentration of higher alcohols (particularly isobutanol) decreased with an increase in original wort gravity, as observed in our study. Furthermore, higher levels of

higher alcohols were observed in ale strains compared to lager strains studied. This is probably due to higher growth level of ale strains and this was pronounced in the strain NCYC1332 (Figure 4.18A), since the biosynthesis of higher alcohols are associated with amino acid metabolism such as Ehrlich pathway (Hazelwood, *et al.*, 2008), also related to cell growth (Quain & Duffield, 1985). Furthermore, it should be noted that high levels of vicinal dikenones (VDK) including diacetyl and 2,3-pentanedione remain a problem in many brewing fermentation system (Branyik, *et al.*, 2005, Willaert & Nedovic, 2006, Verbelen, *et al.*, 2008, Verbelen, *et al.*, 2009). Although they were not the focus of the current study, production of this compound warrants a more thorough investigation in the future.

4.3.2 The impact of wort gravity and pitching rate on yeast replication and physiology

Yeast physiological activity is instrumental in achieving consistent fermentation, leading to beers of acceptable quality. The results presented here show that a higher initial wort density and pitching rate has a strong effect on the growth of brewing yeast cells. Nevertheless, the influence appeared to be strain-dependent. Whilst the growth of ale strain NCYC1332 was greatly enhanced at high gravities, it was interesting to note that lager yeast W34/70 generated a similar number of yeast cells under all conditions except for 24P24M. This result suggested that increasing wort gravity alone does not always result in accelerated cell division, whereas pitching rate is more likely to affect the cell growth of specific strains at high gravities. Verbelen, *et al.* (2008) observed similar amounts of cell growth by comparing low cell density (2.0×10^7 viable cells/mL) and high cell density (8.0×10^7 viable cells/mL) fermentations in high gravity (15 °P) wort. However, the same group also reported that cell growth decreased with increasing pitching rate (1.0, 2.0, 4.0, 8.0 and 1.2×10^7 viable cells/mL) within 15 °P fermentations (Verbelen, *et al.*, 2009). Such contradictions may potentially be attributed to differences between yeast strains, however it is also likely that the

different growth responses can be explained by the depletion of growth limiting factors such as lipids and molecular oxygen.

Biosynthesis of lipids including sterols and unsaturated fatty acids (UFAs) are essential for plasma membrane integrity and the major role of oxygen is to promote the build-up of these compounds during the aerobic phase of fermentation (David & Kirsop, 1973, Alexandre, *et al.*, 1994, Rosenfeld, *et al.*, 2003). Whilst the addition of oxygen in the form of air can be employed in traditional brewing, the use of wort oxygenation is recommended for HG and VHG fermentations (Boulton & Quain, 2001). In order to mimic industrial practice as close as possible, in the current study, an oxygenation regime was applied to HG (18 °P) and VHG (24 °P) worts, which were expected to be ultimately saturated with oxygen, resulting in the dissolved oxygen (DO) levels being 38 and 35 ppm, respectively, whilst the standard gravity (13 °P) wort was expected to be air-saturated to achieve a DO level of 9 ppm. These DO values were established according to the oxygen solubility in wort at each specific gravity and working temperature (Boulton & Quain, 2001), although they were not directly determined due to the limitation of available DO probe. In this case, it could be argued that the increased cell growth at higher gravities simply reflects the higher supply of wort oxygen, irrespective of the availability of wort carbon.

One the other hand, it should be noted that the inoculum preparation method used in this study (Section 2.6.4) was markedly different to that in general brewing practice, given that the yeast used here was under aerobic condition prior to pitch, whereas the yeast used for brewery fermentation was usually exposed to anaerobic environment resulting from yeast recycling practice (Section 1.1.2.3). The likely impact of this distinctly different physiology on the fermentation studies needs to be considered: (I) during initial aerobic fermentation, the available oxygen could be higher than the DO values as described above, which was likely to

increase yeast growth level and extent the period that the oxygen exited in the wort; (II) during the end of yeast propagation, the synthesis of cellular sterols could occur, given the ingredients, glycogen (providing carbon and energy) and oxygen (catalysing reactions), were available for cells. This may provide an explanation for the missing breakdown period in glycogen profiles at the beginning of fermentations (Figures 4.23-4.24); (III) since yeast propagation was conducted aerobically, the oxidative stress induced (Section 1.5) may be lower when the cells were transferred from the aerobic vessel to the aerated or oxygenated wort, compared to brewery practice, where the anaerobic yeast was exposed directly to the oxidative environment. Irrespective, the potential impact of the procedure on yeast physiology and fermentation performance should be identical in each condition. The results obtained indicate that a specific pitching rate should be selected for each strain, allowing an acceptable amount of growth for each set of conditions. The specific initial cell density is likely to be strain-dependent and should be optimised taking into consideration the oxygen requirements of the yeast strain, as well as other process parameters such as wort composition and temperature (Verbelen, *et al.*, 2009).

The fact that cell viability decreased during the course of fermentation was in agreement with the findings of Dekoninck, *et al.* (2012). These authors found that the viability of a lager yeast strain in 15 °P wort remained high, whereas 18 °P fermentations yielded either moderate or drastic decrease in yeast viability. In the current study, a significant reduction in viability was observed at high gravities for all yeast strains, most likely due to the elevated environmental stress conditions associated with HG and VHG fermentations. Despite this, the results indicate that lager strains had a greater ability to survive at higher gravity than the ale strains. Confusingly, Pratt, *et al.* (2003) suggested that ale strains were more tolerant to stress factors associated with high gravity brewing than lager strains. Furthermore, another study has indicated that there were no significant trends observed when comparing ale and lager

yeast viability during high gravity fermentations (Yu, *et al.*, 2012). Consequently, it remains unclear how the stress response influence yeast survival and, furthermore, any potential effects appear to be perhaps strain-dependent rather than group-specific. Because of this, identification of yeast strains with increased tolerance to environmental stresses is of commercial significance to the brewing industry and has been explored at the genomic level. James, *et al.* (2008) examined the chromosome integrity of a stress-tolerant lager yeast strain and its parent during a single round of high gravity fermentation. The authors revealed that exposure to environmental stress could influence chromosomal stability and may result in a high degree of genomic plasticity in lager yeasts (James, *et al.*, 2008). However, analysis of comparative stress tolerance capacity of lager and ale strains under VHG conditions has not yet been fully explored and is the subject of investigation in Chapter 6.

HG and VHG fermentations are believed to have a great influence on the yeast stress response, affecting accumulation of important intracellular carbohydrates trehalose and glycogen (Francois & Parrou, 2001). Trehalose is widely accepted as an important stress protectant, conferring stability to the plasma membrane (Neves, *et al.*, 1991, Mansure, *et al.*, 1994, Petit & Francois, 1994, Plourde-Owobi, *et al.*, 2000, Jules, *et al.*, 2004). In this study, fermentations conducted using standard gravity wort (13 °P) yielded cells which showed only a modest increase in trehalose compared to higher gravities, confirming the general observation that high gravity brewing stimulates the production of trehalose (Gibson, *et al.*, 2007, Piddocke, *et al.*, 2009, Dekoninck, *et al.*, 2012). Apart from this, utilising a higher pitching rate at high gravity resulted in a more rapid accumulation of trehalose in yeast strains Lager1, W34/70 and NCYC1332, when compared to a lower pitching rate. As trehalose concentration can be interpreted as a stress indicator (Majara, *et al.*, 1996b), these results may suggest that increasing of initial cell density may lead to greater stress during the initial stage of fermentation. The enhanced trehalose may help to ensure yeast viability during

fermentation, particularly during the latter stages where yeast essentially encounters a starvation environment, which could potentially results in a reduced fermentation time (Guldfeldt & Arneborg, 1998). Another interesting observation was that yeast strain M2 produced low concentrations of trehalose under all of the different conditions studied. This was surprising and represents a contradiction to previous findings which suggest that trehalose accumulation should be proportional to the original wort gravity (Majara, *et al.*, 1996a, Majara, *et al.*, 1996b). It also indicated that accumulation of trehalose is not an absolutely required characteristic of exposure to adverse conditions; inevitably other factors may play a significant role, including the presence of other potential anti-stress compounds. Furthermore, the precise mechanisms governing the accumulation of trehalose in direct response to increased wort gravity have not yet been elucidated and remain the subject of further investigation.

Intracellular glycogen is regarded as a major storage carbohydrate in yeast, serving as an energy source for maintaining cellular functions and for cell survival, especially during the storage of pitching yeast and during the synthesis of sterols and UFAs (Francois & Parrou, 2001). Previous studies have revealed the complex pattern of glycogen affected by initial cell density (Verbelen, *et al.*, 2009) and wort compositions (Dekoninck, *et al.*, 2012). Verbelen, *et al.* (2009) observed that the typical profile of glycogen was more consistent at higher pitching rates compared to lower pitching rates at 15 °P wort, whilst Dekoninck, *et al.* (2012) showed that the metabolism of glycogen was regulated by different types of sugar adjuncts in 18 °P wort. In the current study, it was interesting to note that the maximum amount of glycogen was not directly influenced by original wort density itself, but was more closely linked to the initial cell concentration for yeast strains Lager1, W34/70 and NCYC1332. In particular it was observed that, at higher gravities (18 °P and 24 °P), employing a standard pitching rate resulted in a higher maximum glycogen concentration when compared to using a higher

pitching rate. These results may indicate that increasing initial cell density could lead to decreased energy stores within yeast cells at the end of fermentation, and consequently further investigation is required to understand the relationship between glycogen production and survival strategies for yeast cells based on initial cell density. Furthermore, another interesting finding was that while glycogen levels declined during the later stages of standard gravity (13P15M) fermentations, HG and VH conditions resulted in relatively consistent glycogen contents. This phenomenon may be explained by a higher degree of glycogen recycling occurring at higher gravities, which is thought to be one of the major effect of stress in yeast (Parrou, *et al.*, 1997).

In order to maintain cellular redox balance, glycerol is produced during fermentations in a redox-neutral process. The synthesis of glycerol serves to consume NADH produced in cellular reactions and promotes ethanol production. Apart from this role, glycerol is believed to be an essential compatible solute during osmoregulation in yeast (Wang, *et al.*, 2001). Elevated formation of glycerol can result from activation of the HOG pathway (Hohmann, 2002) upon osmotic stress exposure. In this study, the formation of glycerol increased with elevated initial wort gravity. It is suggested that this may have been primarily due to the observed increase in ethanol production, with glycerol concomitantly acting as a redox-balancing mediator. Whilst glycerol production could also result from the yeast osmotic stress response, the combination effect was difficult to achieve here. However, the effect of osmotic pressure on glycerol accumulation will be discussed in more detail in Chapter 6. Interestingly, of the yeast strains studied, it was noted that yeast strain Lager1 generated significantly higher amounts of glycerol at higher pitching rates when compared to the standard rates (18P18M to 18P15M, and 24P24M to 24P15M). This unexpected result may be explained by: (I) the surplus formation of NADH in the yeast metabolic pathway, which is responsible for glycerol synthesis; (II) the higher osmotic induction level in the yeast,

indicating the environment is more challenging for this strain, although further analysis is required to substantiate this hypothesis and to determine if this is a species or strain-specific phenomenon.

4.4 Conclusion

In this chapter the influence of HG and VHG brewing on fermentation performance and yeast physiology was investigated in a series of lab-scale fermentations using both lager and ale brewing yeast strains. With a ‘standard’ pitching rate (1.5×10^7 viable cells/mL, irrespective of initial wort Plato), the increased wort gravity (13 °P, 18 °P and 24 °P) resulted in decreased cell viability, slower fermentation rate and incomplete sugar utilisation (mostly maltotriose). Production of ethanol, carbon dioxide and glycerol occurred at a concentration broadly proportional to the original wort gravity, whereas the maximum glycogen produced was apparently not affected by wort density, and the accumulation of trehalose was strain-specific.

The data generated here also allowed comparison of the effects of an adjusted pitching rate (1.0×10^6 viable cells/mL per Plato) to the ‘standard’ pitching rate at high gravities (18 °P and 24 °P). The results obtained provide evidence that the application of the former pitching rate at 24 °P brewing can not only function to accelerate the fermentation process, but may also result in a more complete carbohydrate utilisation, leading to enhanced ethanol yield without serious alterations in flavour synthesis. The adjusted pitching rate was generally regarded as ‘rule of thumb’ in brewing practice, and the results indicate the benefits of making adjustments centred around this principle for VHG fermentations. In addition, this study also highlighted the significance of yeast pitching rate with respect to yeast physiology and fermentation efficiency. It is proposed that a specific pitching rate should be optimised for individual brewing strains at high gravity, allowing the brewer to control the extent of cell growth and ultimately leading to optimisation of fermentation efficiency.

**CHAPTER 5: THE EFFECT OF WORT SUGAR
CONCENTRATION ON YEAST CARBON
PARTITIONING DURING BREWING
FERMENTATIONS**

5.1 Introduction

Management of ethanol production is of great importance to the brewing industry, as well as related sectors such as oenology and distilling, in order to maximise fermentation efficiency (Vargas, *et al.*, 2011). This is particularly relevant for High Gravity (HG) and Very High Gravity (VHG) brewing fermentations, which employ highly concentrated substrates primarily to obtain a greater total ethanol yield than normal (Section 1.3.6). Brewing yeast acts as the workhorse in this reaction, converting wort components to ethanol and flavour compounds. However, sugars are also required for a number of other biological processes including growth and division as well as for cellular homeostasis, which incorporates the maintenance of redox balance, generation of energy, production of storage carbohydrates and activation of anti-stress pathways (Section 1.4.5). As a result, yeasts are capable of producing a range of carbon metabolites including ethanol, carbon dioxide, glycerol, trehalose, glycogen, higher alcohols and esters, as well as polysaccharides (including glucan and mannan) and lipid structures used for yeast biomass production (Figure 5.1).

It is known that the use of highly concentrated worts and the transition to a high ethanol environment (during HG and VHG brewing) can have a profoundly negative effect on yeast quality and fermentation performance (Casey, *et al.*, 1984, Pidcocke, *et al.*, 2009, Sigler, *et al.*, 2009, Dekoninck, *et al.*, 2012, Lei, *et al.*, 2012, Zhuang, *et al.*, 2012, Zhuang, *et al.*, 2013). In order to eliminate or reduce such effects, yeast cells respond to their environment by shunting carbon into different metabolic end products as described above. Some of these metabolites can assist in the protection of cells, but the diversion of carbon can also impact on final ethanol yield. Consequently, the way in which brewing yeast adapt their central carbon flux in response to the wort environment is critical in determining both fermentation efficiency and the health of the yeast culture.

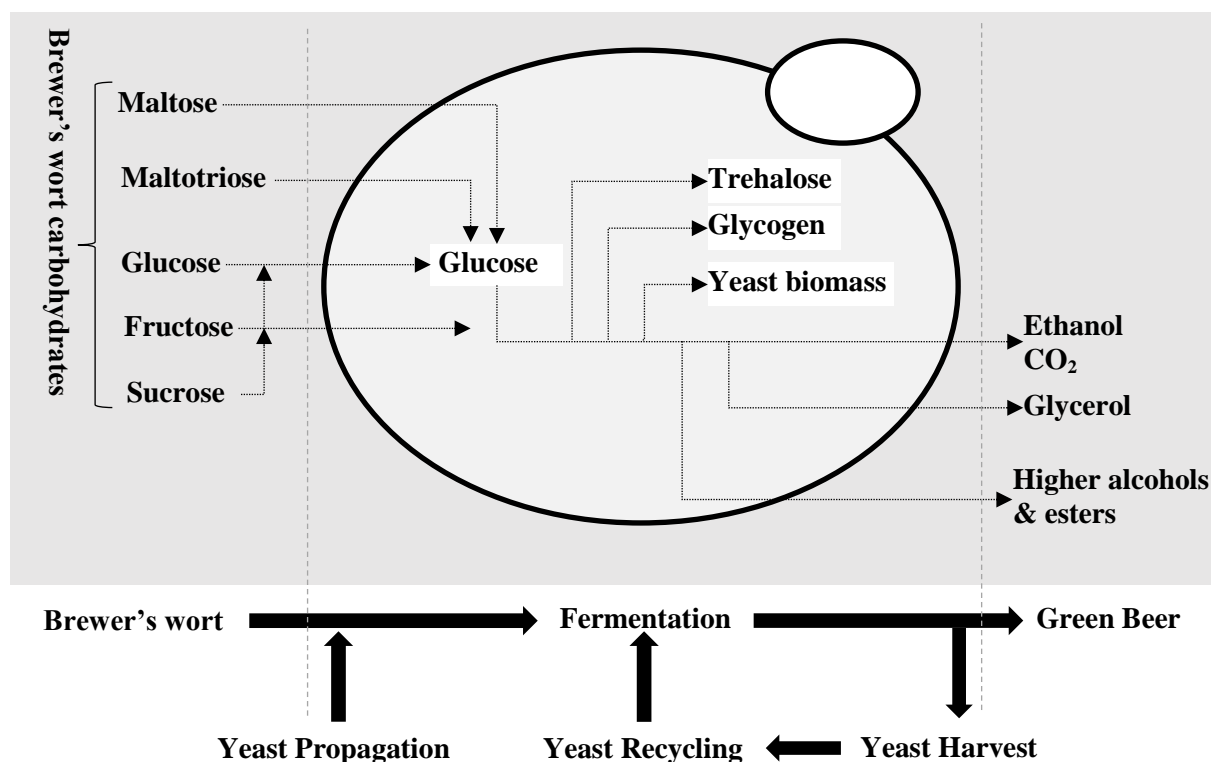


Figure 5.1 Overview of central carbon metabolism from brewer's wort carbohydrates to major carbon metabolites of brewing yeast.

Typical approaches to quantifying carbon distribution are based on mass balance analysis. Antoine Lavoisier first described this in 1790 based on the realization that sugars are transformed into carbonic acid, alcohol, and yeast biomass (Lavoisier, 1790). Subsequently Karl Napoleon Balling published a fermentation mass balance formula on the basis of the concept that fermentable wort solids contribute to yeast mass increase. This formula has been applied in brewing practice for over 100 years (De Clerck, 1958, Nielsen, 2004) and is accepted as standard by the American Society of Brewing Chemists (Beer-6A, 2014). However, two assumptions are made in the derivation of Balling's formula: (I) 0.11 g of carbohydrate is converted to yeast mass for each gram of ethanol produced in fermentation; (II) all fermentable dissolved wort solids are monosaccharides. These assumptions are not

wholly justified based on current knowledge of yeast metabolism and wort composition during brewing fermentations. Cutaia (2007) compared stoichiometric values to Balling's classic formula during a brewing fermentation, taking into consideration the major wort carbohydrates (glucose, fructose, maltose and maltotriose) and factors associated with yeast growth including sterols and dissolved oxygen. The author concluded that a significant departure from the traditional wort profile, such as the application of high gravity brewing, could result in inaccurate estimates using Balling's original formula. Consequently, while significant insights into the subject in general have been made, the apportioning of carbon contribution to yeast metabolites under HG and VHG conditions has not been fully explored.

This study aims to evaluate the carbon partitioning of brewing lager and ale yeast under different fermentation conditions, and to highlight potential strategies for management of ethanol content at higher gravity fermentations. Two assumptions were applied adapted from Cutaia (2007) in order to implement this quantification: (I) carbon conversion from fermentable carbohydrates (sugars) was regarded as total carbon input, whilst the assimilation of non-carbohydrate materials from brewer's wort, such as free amino nitrogen, was considered negligible; (II) carbon conversion to fermentation products other than ethanol, carbon dioxide, glycerol, yeast biomass, glycogen, trehalose, higher alcohols and esters, was considered to be negligible, such as organic acid. In this fashion a measurement of carbon distribution was developed by calculation of carbon assimilation and dissimilation around yeast central carbon flux.

5.2 Results

To determine the relationship between wort gravity and yeast carbon partitioning, commercial worts of 13 °P and 25 °P were obtained from Molson Coors Brewing Company (Burton-on-Trent, UK). The former was used directly, whilst the latter was used to prepare

HG (18 °P) and VHG (24 °P) worts as described previously (Section 2.6.1). Each wort was fermented using lab-scale miniature fermenters (100 mL working volume) in conjunction with two lager and two ale strains as described in Section 2.6.3. Samples were taken at the start (0 hour) and end point (120 hour) of each set of fermentation to determine both the carbon concentration of wort carbohydrates consumed (Section 5.2.1) and the carbon metabolites produced (Section 5.2.2).

It should be noted that the five different experimental conditions examined previously were used here, with adjustments to oxygen condition and pitching rate as described in Section 2.6 and Chapter 4. Briefly, 13 °P fermentations were pitched at 1.5×10^7 viable cells/mL (13P15M); 18 °P fermentations were pitched at both 1.5×10^7 viable cells/mL (18P15M) and 1.8×10^7 viable cells/mL (18P18M); 24 °P fermentations were pitched at 1.5×10^7 viable cells/mL (24P15M) and 2.4×10^7 viable cells/mL (24P24M). HG (18 °P) and VHG (24 °P) worts were oxygenated, whilst standard gravity (13 °P) wort was aerated for a couple of hours prior to pitching as described in Section 2.6.2. The experimental programme was conducted in sequence.

5.2.1 Estimation of total carbon input

The concentration of each carbohydrate in the original wort and the final beers was determined using high-performance liquid chromatography (HPLC) (Section 2.7.2). Carbohydrate consumption (original wort carbohydrate content minus final beer carbohydrate concentration) was calculated for each set of fermentations and included analysis of monosaccharides (fructose and glucose), disaccharides (sucrose and maltose) and a trisaccharide (maltotriose). Subsequently this value was used to quantify total carbon input (assimilation by yeast) on the basis of carbon conservation within each group, representing

the sum of the carbon content of wort carbohydrates consumed (Section 2.9). Data indicating carbohydrate utilisation and total carbon input is summarized in Table 5.1.

The utilisation of monosaccharides, disaccharides and trisaccharide is depicted in Figures 5.2-5.4, respectively. As expected, for each group of sugars, consumption increased with increasing wort density, and no significant difference was found between the four strains for either the consumption of monosaccharides (Figure 5.2) or disaccharides (Figure 5.3). However, it was interesting to note that at 24P15M, the ale strain NCYC1332 utilized a lower amount of trisaccharide (maltotriose) than the other yeasts (Figure 5.4). Nevertheless, this strain exhibited higher ($P < 0.05$) maltotriose consumption at 24P24M compared to 24P15M condition and under 24P24M condition the utilisation level was similar to the other strains investigated (Figure 5.4).

Corresponding to the data reported above, the total carbon input increased with elevated original wort gravity, with approximately 39 g/L, 51 g/L and 72 g/L for 13 °P, 18 °P and 24 °P fermentations, respectively (Figure 5.5). At each condition, no significant difference in total carbon input ($P < 0.05$) was found for the yeast strains investigated, except for ale yeast NCYC1332 at 24P15M, which consumed a significantly ($P < 0.05$) lower amount of carbon overall than the other strains investigated (Table 5.1), likely to be directly related to the relative lower consumption of maltotriose described previously (Figure 5.4).

Table 5.1 Carbohydrate utilisation and total carbon input under different fermentation conditions. The concentration of each carbohydrate was assessed using HPLC analysis. Data represents the mean of triplicate analyses \pm the standard deviation.

Strains	Carbohydrate utilisation (g/L) ^a			Total carbon input (g/L) ^b
	Monosaccharides	Disaccharides	Trisaccharide	
13P15M				
Lager1	12.8 ± 1.1	59.8 ± 2.3	19.5 ± 2.1	38.6 ± 1.9
W34/70	12.9 ± 1.5	59.6 ± 1.4	19.4 ± 0.6	38.7 ± 1.8
NCYC1332	12.9 ± 1.3	59.8 ± 2.3	19.5 ± 1.1	38.7 ± 1.5
M2	12.8 ± 2.1	59.8 ± 1.1	19.5 ± 1.4	38.6 ± 2.2
18P15M				
Lager1	17.7 ± 3.1	81.1 ± 3.5	23.6 ± 3.1	51.1 ± 2.3
W34/70	17.3 ± 2.2	81.5 ± 1.7	23.6 ± 2.4	50.1 ± 2.9
NCYC1332	17.1 ± 1.5	81.3 ± 4.2	23.7 ± 2.1	50.1 ± 2.6
M2	17.2 ± 4.1	80.8 ± 2.1	23.5 ± 1.2	50.9 ± 1.9
18P18M				
Lager1	17.4 ± 2.5	81.8 ± 5.1	24.8 ± 2.2	52.0 ± 2.5
W34/70	17.4 ± 1.8	81.9 ± 2.4	24.4 ± 1.2	51.8 ± 2.5
NCYC1332	17.2 ± 2.2	81.8 ± 3.7	24.5 ± 1.3	51.8 ± 2.4
M2	17.3 ± 2.5	82.2 ± 2.5	24.7 ± 2.1	52.1 ± 2.5
24P15M				
Lager1	25.1 ± 3.1	110.0 ± 1.3	30.7 ± 1.5	68.5 ± 2.0
W34/70	25.6 ± 5.3	109.9 ± 2.2	31.6 ± 4.1	69.8 ± 1.4
NCYC1332	25.0 ± 2.7	107.1 ± 1.4	28.5 ± 2.0	66.3 ± 1.5
M2	25.2 ± 7.5	109.3 ± 1.3	30.4 ± 1.1	69.0 ± 1.8
24P24M				
Lager1	25.2 ± 3.1	111.7 ± 2.3	33.6 ± 3.3	71.5 ± 2.2
W34/70	25.5 ± 2.2	111.8 ± 2.2	33.7 ± 2.1	71.6 ± 0.9
NCYC1332	25.7 ± 2.7	111.5 ± 1.1	32.9 ± 4.1	71.1 ± 1.1
M2	25.1 ± 1.9	111.3 ± 2.5	32.9 ± 1.8	71.1 ± 1.4

^a Carbohydrate utilisation = original wort carbohydrate content – final beer carbohydrate content. Data represents the concentration of each carbohydrate group: monosaccharides (fructose and glucose), disaccharides (sucrose and maltose), and trisaccharide (maltotriose).

^b Total carbon input was the sum of carbon utilisation from every carbohydrate group. For each group, carbon utilisation was quantified based on carbon conservation of the utilized carbohydrate, determined from the carbohydrate values of original wort and final beer.

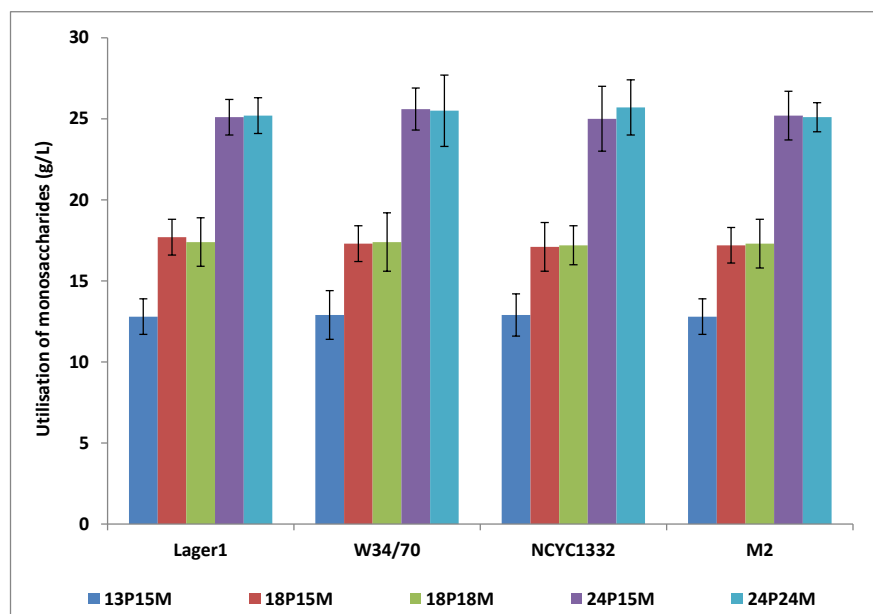


Figure 5.2 Concentration of monosaccharides (fructose and glucose) utilized under different fermentation conditions. Utilisation was determined based on the carbohydrate values of the original wort and final beer by HPLC. Data represents the mean of triplicate analyses, with error bars showing the standard deviation.

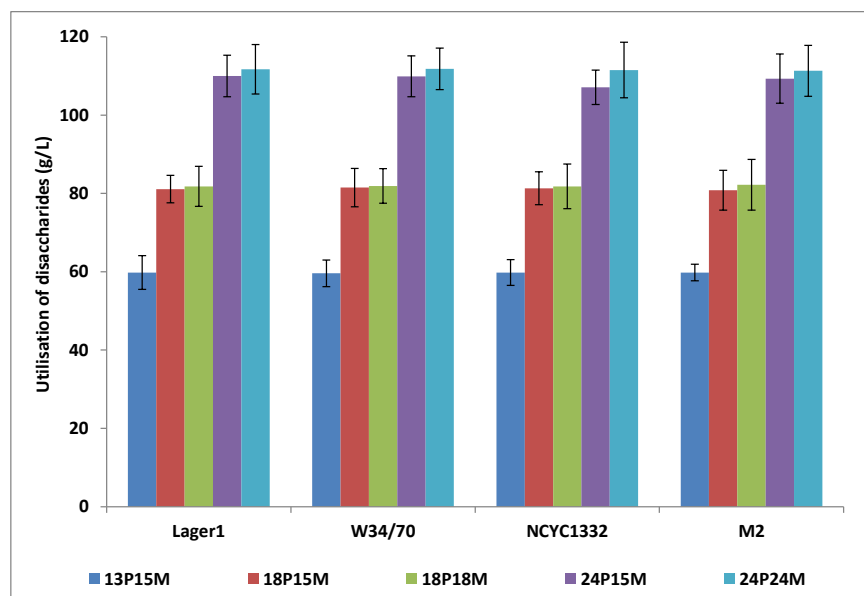


Figure 5.3 Concentration of disaccharides (sucrose and maltose) utilized under different fermentation conditions. Utilisation was determined based on the carbohydrate values of the original wort and final beer by HPLC. Data represents the mean of triplicate analyses, with error bars showing the standard deviation.

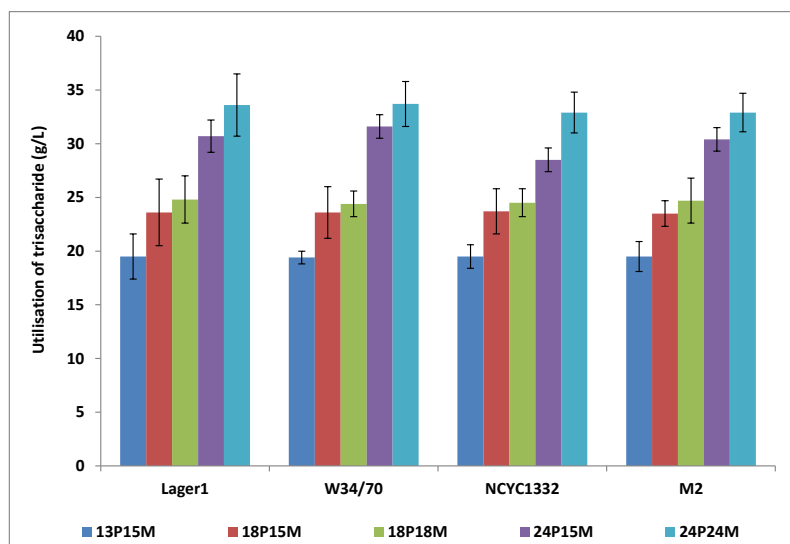


Figure 5.4 Concentration of trisaccharide (maltotriose) utilized under different fermentation conditions. Utilisation was determined based on the carbohydrate values of the original wort and final beer by HPLC. Data represents the mean of triplicate analyses, with error bars showing the standard deviation.

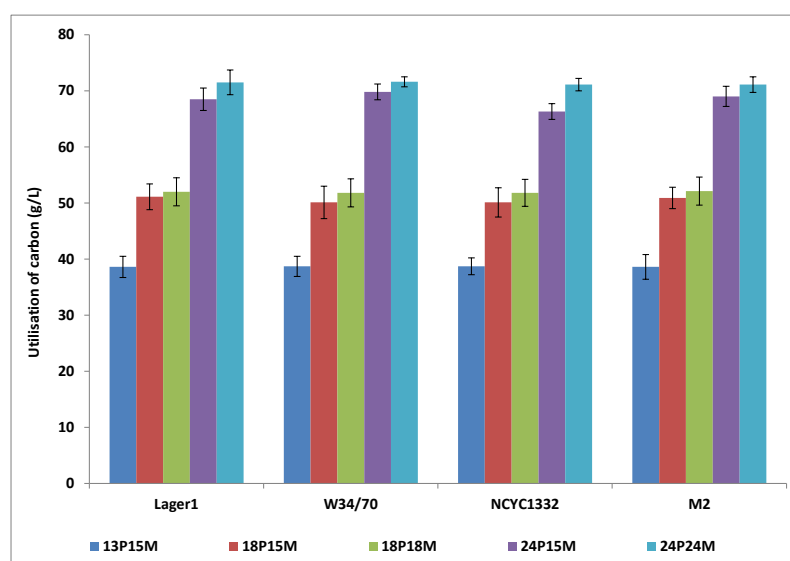


Figure 5.5 Concentration of total carbon input under different fermentation conditions. Total carbon input was the sum of carbon utilisation from monosaccharides, disaccharides and trisaccharide. For each group, carbon utilisation was quantified based on carbon conservation of utilized carbohydrate, determined from the carbohydrate values of original wort and final beer by HPLC. Data represents the mean of triplicate analyses, with error bars showing the standard deviation.

5.2.2 Estimation of carbon output in the form of carbon metabolites

Concentration of each carbon product was obtained from the values obtained at the start (0 hour) and end point (120 hour) of each set of fermentations, using methods described in Sections 2.8-2.9. Briefly, concentration of ethanol and carbon dioxide were determined by HPLC (Section 2.7.3) and total weight loss (Section 2.6.6), respectively. Concentration of higher alcohols and esters were determined using headspace GC-MS (Section 2.7.4), and glycerol content was determined using HPLC (Section 2.7.3). Intracellular trehalose and glycogen were broken down into glucose enzymatically and assayed using a commercial glucose kit (Section 2.8.1). Subsequently, the carbon content of each compound was calculated using derived equations (Section 2.9.1) on the basis of carbon conservation of individual components. The total carbon input obtained (Section 5.2.1) was used to quantify carbon investment in each metabolite (Section 2.9.2). Data are expressed as a percentage of the total carbon input and are summarized in Tables 5.2-5.5 for each brewing yeast strain.

Ethanol occupied the most abundant form of carbon output in all fermentations, representing greater than 50 % of the total carbon input (Figure 5.6). At 24 °P fermentations, a higher amount of carbon ($P < 0.05$) in the form of ethanol was observed at higher pitching rates (24P24M compared to 24P15M) for each of the strains examined, except for lager strain W34/70, which exhibited similar carbon investment in ethanol under both conditions (Figure 5.6). At 18 °P, only strain NCYC1332 showed a higher carbon to ethanol conversion at the higher pitching rate (18P18M compared to 18P15M) ($P < 0.05$), whilst no significant difference ($P < 0.05$) was observed for the other strains (Figure 5.6). Moreover, the ale strain NCYC1332 displayed the lowest carbon percentage in the form of ethanol under each set of fermentation conditions, whereas the lager strain W34/70 showed the highest value among the four strains. Estimation of the proportion of carbon attributed to carbon dioxide provided comparable results; carbon percentages in ethanol and carbon dioxide were present in ca. 2/1

ratio as expected (Tables 5.2-5.5). This is unsurprising since the total ethanol yield and carbon dioxide evolved correlates well (a correlation coefficient of > 0.99) at each set of fermentations (Section 4.2.1.4). The 2/1 ratio was derived based on the theoretical reaction equation that 1 molecule of glucose ($C_6H_{12}O_6$) was converted to 2 molecule of ethanol (C_2H_6O) and 2 molecule of CO_2 , where the carbon in the form of ethanol and CO_2 was represent in a ratio of 2/1.

Analysis of carbon to glycerol indicated that only 2-4 % of carbon was directed towards the production of this molecule (Figure 5.7), and the percent output in response to different conditions varied between the strains independent of wort gravity and pitching rate. The exception to this was strain Lager1, which directed higher ($P < 0.05$) amounts of carbon into glycerol at higher pitching rates (18P18M and 24P24M) than when pitching lower numbers of cells (18P15M and 24P15M) (Figure 5.7). In addition, significantly less carbon was directed into trehalose and glycogen, representing approximately 0.1-0.2 % and 0.2-0.3 % of the total carbon input for higher and lower pitching rates respectively (Tables 5.2-5.5). With regard to trehalose, it was interesting to note that both the lager strains directed higher amounts of carbon into trehalose at 24P24M conditions than at 24P15M, whereas the ale strains did not. In contrast, all strains (lagers and ales) directed similar amount of carbon into trehalose at 18P15M and 18P18M conditions. With respect to glycogen, in comparing 13P15M, 18P15M and 24P15M conditions, only the ale strain M2 showed a decreased allocation of carbon with increasing wort gravity, whereas similar proportions were observed between different conditions in all other strains. In addition, higher amounts of carbon in the form of glycogen were observed at 18P15M than at 18P18M conditions, irrespective of yeast strain. In contrast, carbon investment in glycogen was strain dependent when comparing different pitching rates in VHG worts (24P15M and 24P24M conditions).

Approximately 0.2-0.3 % of the total carbon input was diverted to the production of higher alcohols and esters in the four yeast strains examined (Tables 5.2-5.5). In comparing 18P15M and 24P15M, strain W34/70 directed slightly higher amounts of carbon into these flavour compounds at 24 °P than 18 °P fermentations whereas the carbon investment of the other strains was not affected by wort density (18 or 24 °P). Additionally, analysis of carbon to flavour compounds indicated that there was no significant difference either between 18P18M and 18P15M or between 24P24M and 24P15M, regardless of yeast strains (Tables 5.2-5.5).

Carbon proportion attributed to yeast biomass was estimated in two ways. Initially, carbon products other than ethanol, carbon dioxide, glycerol, trehalose, glycogen, higher alcohols and esters were included in the ‘un-allocated’ portion of the total carbon input and this carbon was attributed to yeast biomass production (yeast biomass 1, Tables 5.2-5.5), produced as an artefact of cell maintenance, growth and division based on the observations of Cutaia (2007). In addition, the carbon investment towards yeast biomass was calculated from the increase of yeast dry mass at the end of each set of fermentation, based on the carbon content in dried wine yeast of 48 % (Rosen, 1989) (Section 2.9.2) and expressed as a percentage of the total carbon input (yeast biomass 2; Tables 5.2-5.5). Although there are variations between these two estimations, they yielded broadly comparable data. At 24 °P fermentations, the proportion of carbon in the form of biomass was observed to be lower ($P < 0.05$) when pitching rate was increased (24P24M compared to 24P15M) for all yeast strains except for W34/70, which showed a similar carbon flow into yeast biomass (Table 5.3). In contrast, at 18 °P fermentations, the values were similar ($P < 0.05$) irrespective of pitching rate for both lager strains and one of the ale strains. The exception being the ale strain NCYC1332 which displayed a lower carbon percentage in the form of biomass at 18P18M when compared to 18P15M (Table 5.4). Consequently, the effect of wort gravity and pitching rate on biomass production appeared to be strain-specific, however an overall trend was observed indicating a

direct carbon trade-off between ethanol yield and biomass production when plotting the 'biomass concentration' against 'ethanol concentration' (Figures 5.8-5.9). This is supported by data indicating that the majority of strains investing in a low carbon to biomass ratio also directed a high proportion of carbon to the formation of ethanol (Figures 5.8-5.9). Furthermore, perhaps the carbon distribution between ethanol and biomass is strain-specific, regardless of fermentation conditions (Figure 10).

Table 5.2 Carbon partitioning of lager strain Lager1 under different fermentation conditions. Data is presented as a percentage of the total carbon input and represents the mean \pm standard deviation of three independent replicates.

<i>Carbon output (Percent of total carbon input) ^a</i>	<i>Fermentation conditions</i>				
	<i>13P15M</i>	<i>18P15M</i>	<i>18P18M</i>	<i>24P15M</i>	<i>24P24M</i>
<i>Ethanol</i>	59.3 \pm 1.0	59.7 \pm 0.5	59.8 \pm 0.3	58.9 \pm 0.5	60.8 \pm 0.3
<i>Carbon dioxide</i>	30.8 \pm 0.7	30.6 \pm 0.2	29.9 \pm 0.5	30.5 \pm 0.3	29.8 \pm 0.5
<i>Glycerol</i>	3.0 \pm 0.2	2.8 \pm 0.1	3.5 \pm 0.2	2.5 \pm 0.1	3.5 \pm 0.2
<i>Trehalose</i>	0.07 \pm 0.01	0.06 \pm 0.01	0.06 \pm 0.01	0.09 \pm 0.01	0.17 \pm 0.01
<i>Glycogen</i>	0.23 \pm 0.05	0.32 \pm 0.01	0.22 \pm 0.01	0.30 \pm 0.02	0.23 \pm 0.03
<i>Higher alcohol and ester</i>	0.20 \pm 0.02	0.22 \pm 0.04	0.22 \pm 0.02	0.21 \pm 0.01	0.20 \pm 0.03
<i>Yeast biomass 1 ^b</i>	6.4 \pm 0.9	6.3 \pm 0.3	6.3 \pm 0.9	7.5 \pm 0.5	5.3 \pm 0.4
<i>Yeast biomass 2 ^c</i>	6.6 \pm 0.4	6.1 \pm 0.5	5.9 \pm 0.2	6.9 \pm 0.3	5.7 \pm 0.1

^a Calculations were based on the values obtained from analysis of the compounds at start and end point of each set of fermentations.

^b Data comprises un-allocated carbon proportion of the total carbon input, assuming that this percentage of carbon was attributed to yeast biomass, most likely as an artefact of cell maintenance, growth and division.

^c Data was calculated from the increase of yeast dry weight at the end of each set of fermentations, based on a carbon content in dry yeast biomass of 48 %.

Table 5.3 Carbon partitioning of lager strain W34/70 under different fermentation conditions. Data is presented as a percentage of the total carbon input and represents the mean \pm standard deviation of three independent replicates.

<i>Carbon output (Percent of total carbon input) ^a</i>	<i>Fermentation conditions</i>				
	<i>13P15M</i>	<i>18P15M</i>	<i>18P18M</i>	<i>24P15M</i>	<i>24P24M</i>
<i>Ethanol</i>	59.8 \pm 0.6	60.1 \pm 0.7	60.4 \pm 0.7	60.2 \pm 0.5	60.7 \pm 0.4
<i>Carbon dioxide</i>	29.7 \pm 0.3	30.2 \pm 0.3	30.2 \pm 0.2	30.1 \pm 0.2	29.6 \pm 0.2
<i>Glycerol</i>	2.9 \pm 0.3	2.8 \pm 0.1	2.5 \pm 0.1	2.2 \pm 0.3	2.3 \pm 0.2
<i>Trehalose</i>	0.08 \pm 0.01	0.08 \pm 0.02	0.07 \pm 0.01	0.06 \pm 0.01	0.20 \pm 0.01
<i>Glycogen</i>	0.25 \pm 0.04	0.27 \pm 0.03	0.23 \pm 0.01	0.21 \pm 0.03	0.29 \pm 0.01
<i>Higher alcohol and ester</i>	0.17 \pm 0.03	0.15 \pm 0.02	0.20 \pm 0.03	0.23 \pm 0.03	0.21 \pm 0.02
<i>Yeast biomass 1 ^b</i>	7.1 \pm 0.8	6.4 \pm 0.7	6.4 \pm 0.2	7.0 \pm 0.2	6.7 \pm 0.4
<i>Yeast biomass 2 ^c</i>	7.5 \pm 0.5	6.6 \pm 0.3	6.3 \pm 0.5	6.6 \pm 0.4	6.4 \pm 0.6

^a Calculations were based on the values obtained from analysis of the compounds at start and end point of each set of fermentations.

^b Data compromises un-allocated carbon proportion of the total carbon input, assuming that this percentage of carbon was attributed to biomass, most likely as an artefact of cell maintenance, growth and division.

^c Data was calculated from the increase of yeast dry weight at the end of each set of fermentations, based on a carbon content in dry yeast biomass of 48 %.

Table 5.4 Carbon partitioning of ale strain NCYC1332 under different fermentation conditions.
Data is presented as a percentage of the total carbon input and represents the mean \pm standard deviation of three independent replicates.

<i>Carbon output (Percent of total carbon input) ^a</i>	<i>Fermentation conditions</i>				
	<i>13P15M</i>	<i>18P15M</i>	<i>18P18M</i>	<i>24P15M</i>	<i>24P24M</i>
<i>Ethanol</i>	57.9 \pm 0.7	57.1 \pm 0.7	59.0 \pm 0.4	57.3 \pm 0.5	59.0 \pm 0.2
<i>Carbon dioxide</i>	28.2 \pm 0.6	28.4 \pm 0.4	29.2 \pm 0.5	28.4 \pm 0.3	29.1 \pm 0.2
<i>Glycerol</i>	3.2 \pm 0.2	3.3 \pm 0.3	3.0 \pm 0.3	2.7 \pm 0.2	2.8 \pm 0.2
<i>Trehalose</i>	0.10 \pm 0.01	0.09 \pm 0.02	0.09 \pm 0.01	0.08 \pm 0.01	0.08 \pm 0.01
<i>Glycogen</i>	0.30 \pm 0.05	0.32 \pm 0.01	0.29 \pm 0.01	0.30 \pm 0.01	0.28 \pm 0.01
<i>Higher alcohol and ester</i>	0.30 \pm 0.02	0.29 \pm 0.03	0.32 \pm 0.01	0.29 \pm 0.03	0.26 \pm 0.1
<i>Yeast biomass 1 ^b</i>	10.0 \pm 0.5	10.5 \pm 0.5	8.1 \pm 0.8	10.6 \pm 0.6	8.3 \pm 0.5
<i>Yeast biomass 2 ^c</i>	9.2 \pm 0.6	9.6 \pm 0.4	8.5 \pm 0.5	9.8 \pm 0.3	8.5 \pm 0.5

^a Calculations were based on the values obtained from analysis of the compounds at start and end point of each set of fermentations.

^b Data compromises un-allocated carbon proportion of the total carbon input, assuming that this percentage of carbon was attributed to biomass, most likely as an artefact of cell maintenance, growth and division.

^c Data was calculated from the increase of yeast dry weight at the end of each set of fermentations, based on a carbon content in dry yeast biomass of 48 %.

Table 5.5 Carbon partitioning of ale strain M2 under different fermentation conditions. Data is presented as a percentage of the total carbon input and represents the mean \pm standard deviation of three independent replicates.

<i>Carbon output (Percent of total carbon input) ^a</i>	<i>Fermentation conditions</i>				
	<i>13P15M</i>	<i>18P15M</i>	<i>18P18M</i>	<i>24P15M</i>	<i>24P24M</i>
<i>Ethanol</i>	58.6 \pm 0.2	58.9 \pm 0.5	59.1 \pm 0.5	59.2 \pm 0.4	60.2 \pm 0.3
<i>Carbon dioxide</i>	29.2 \pm 0.3	29.1 \pm 0.5	30.0 \pm 0.3	29.5 \pm 0.1	30.1 \pm 0.7
<i>Glycerol</i>	3.1 \pm 0.1	3.0 \pm 0.5	2.8 \pm 0.2	2.7 \pm 0.2	2.8 \pm 0.1
<i>Trehalose</i>	0.03 \pm 0.01	0.03 \pm 0.01	0.04 \pm 0.01	0.01 \pm 0.01	0.02 \pm 0.01
<i>Glycogen</i>	0.31 \pm 0.02	0.33 \pm 0.01	0.26 \pm 0.02	0.21 \pm 0.01	0.26 \pm 0.01
<i>Higher alcohol and ester</i>	0.16 \pm 0.01	0.24 \pm 0.01	0.30 \pm 0.01	0.28 \pm 0.02	0.32 \pm 0.0
<i>Yeast biomass 1 ^b</i>	8.6 \pm 0.6	8.4 \pm 0.5	7.5 \pm 0.4	8.1 \pm 0.7	6.3 \pm 0.6
<i>Yeast biomass 2 ^c</i>	8.4 \pm 0.4	7.9 \pm 0.7	7.6 \pm 0.3	7.5 \pm 0.6	5.9 \pm 0.5

^a Calculations were based on the values obtained from analysis of the compounds at start and end point of each set of fermentations.

^b Data compromises un-allocated carbon proportion of the total carbon input, assuming that this percentage of carbon was attributed to biomass, most likely as an artefact of cell maintenance, growth and division.

^c Data was calculated from the increase of yeast dry weight at the end of each set of fermentations, based on a carbon content in dry yeast biomass of 48 %.

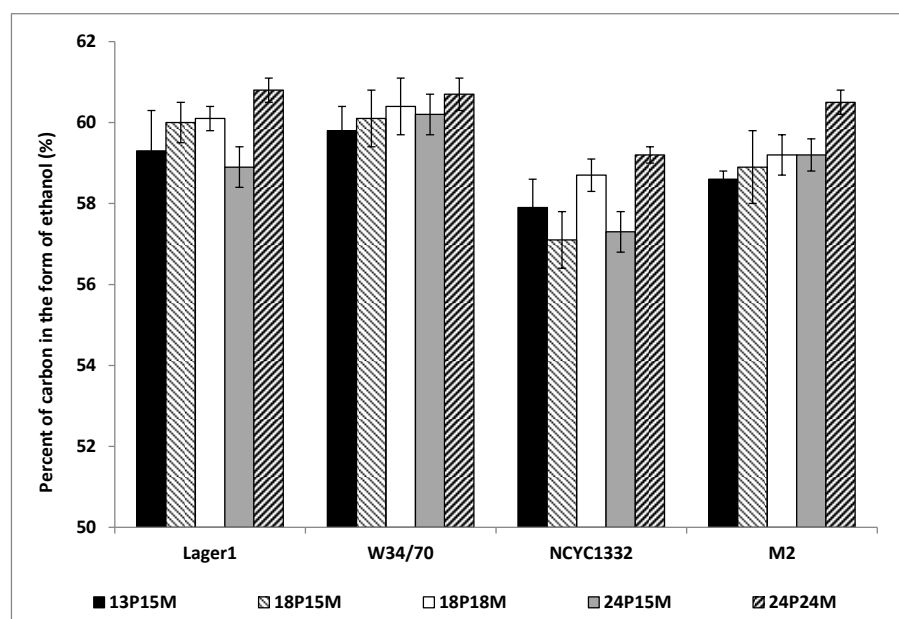


Figure 5.6 Percentage of carbon in the form of ethanol under different fermentation conditions. Data is presented as the percentage of total carbon input (determined from utilized wort carbohydrates) and represents the mean of triplicate analyses, with error bars indicating the standard deviation.

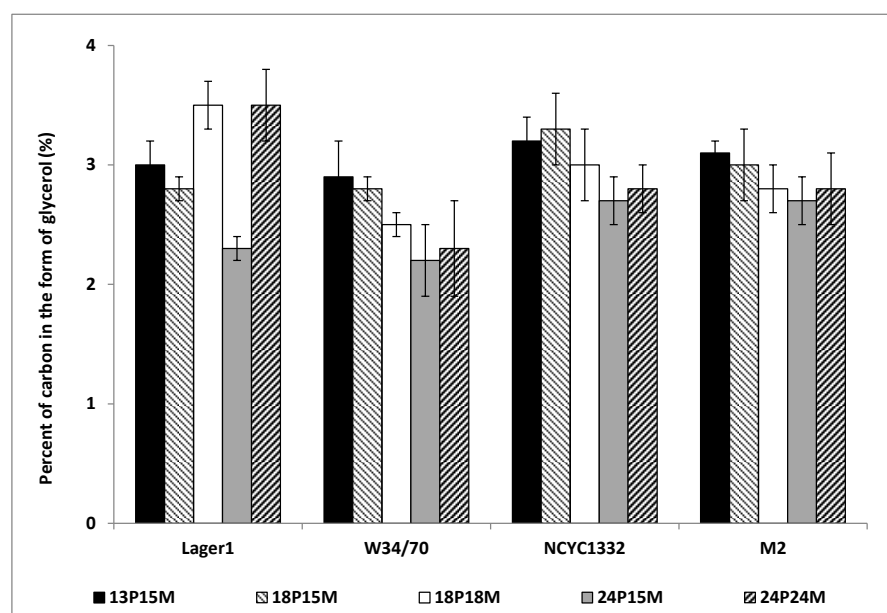


Figure 5.7 Percentage of carbon in the form of glycerol under different fermentation conditions. Data is presented as the percentage of total carbon input (determined from utilized wort carbohydrates) and represents the mean of triplicate analyses, with error bars indicating the standard deviation.

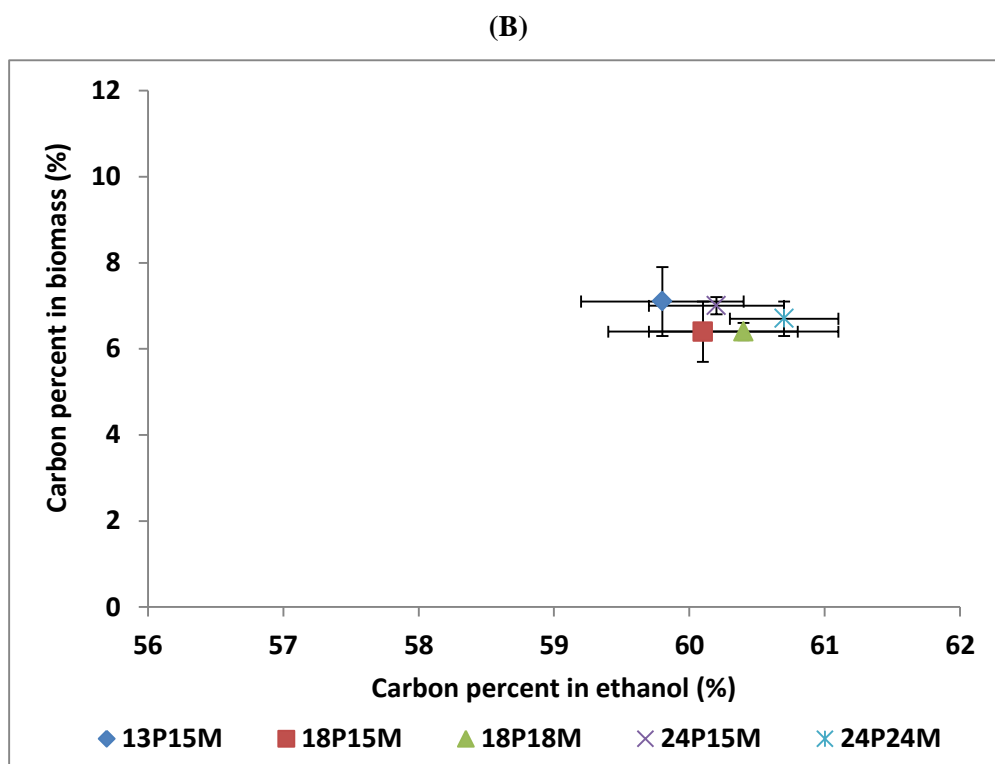
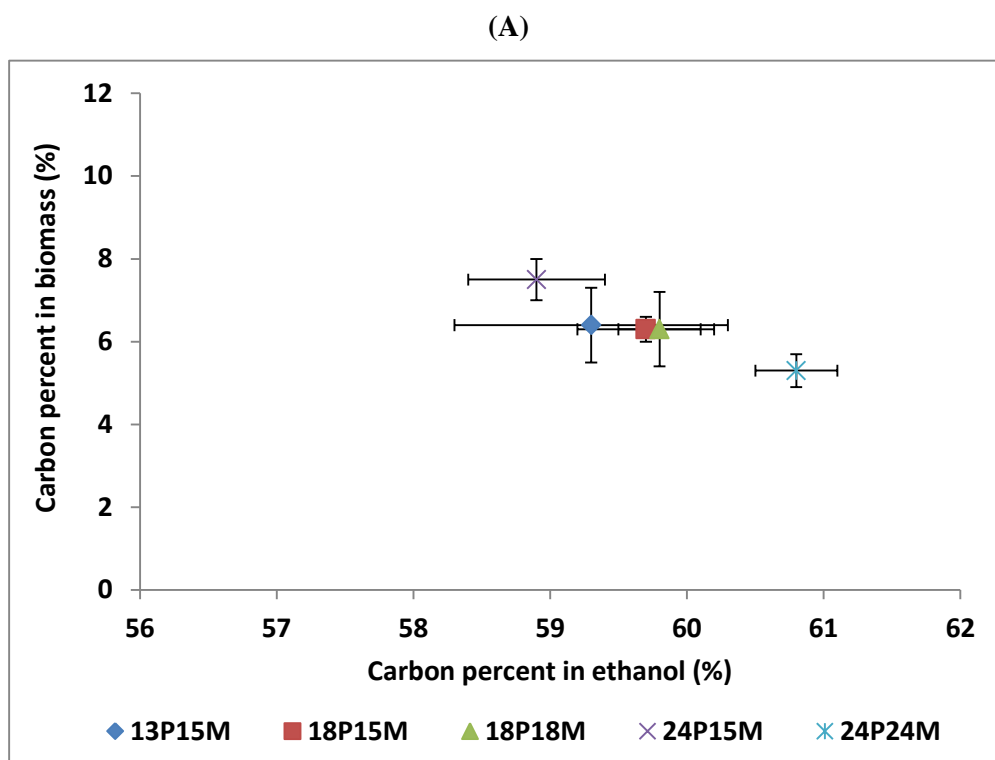


Figure 5.8 Relationship between carbon proportion in the form of biomass and ethanol under different fermentation conditions using lager yeast strains Lager1 (A) and W34/70 (B). Data represents the mean of triplicate analyses, with error bars showing the standard deviation.

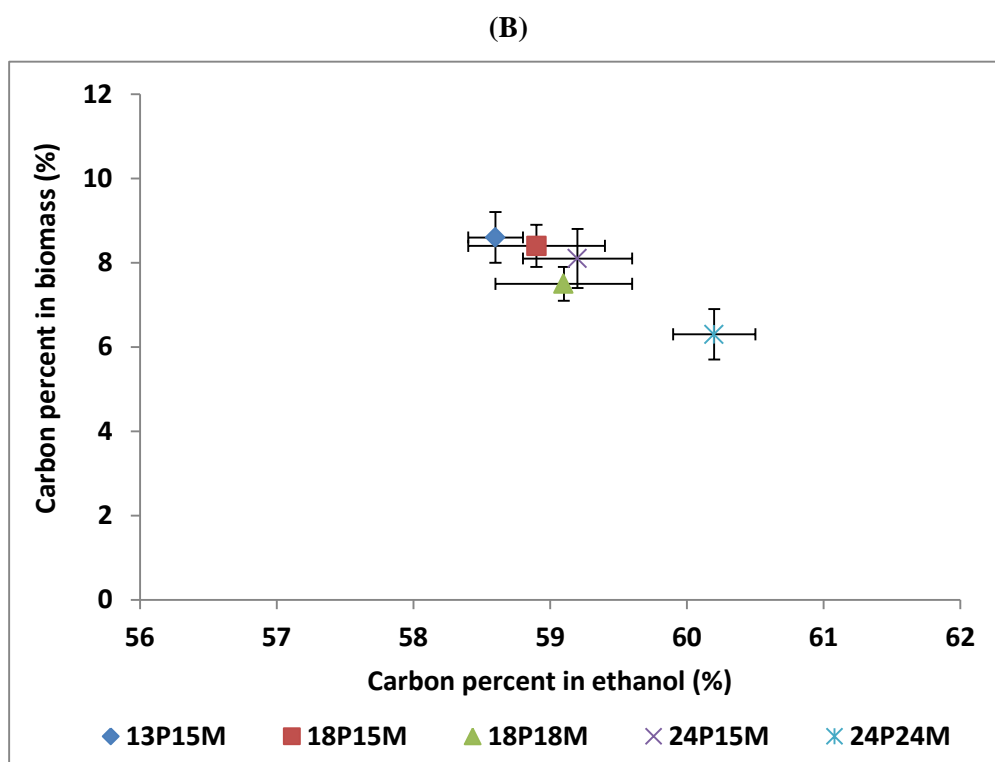
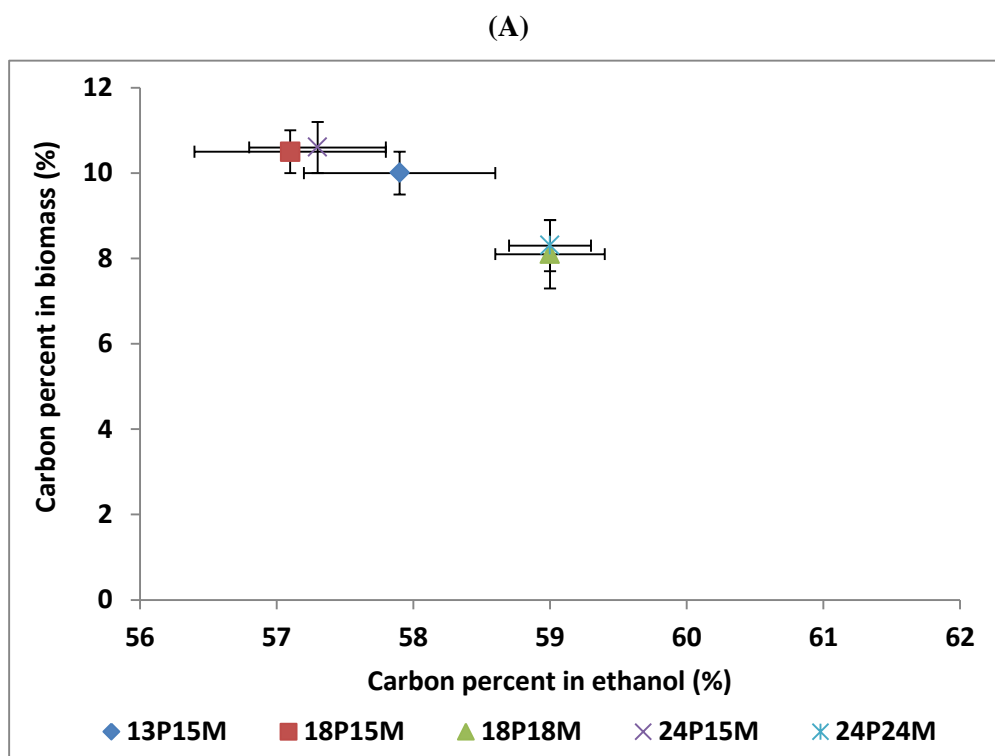


Figure 5.9 Relationship between carbon proportion in the form of biomass and ethanol under different fermentation conditions using ale yeast strains NCYC1332 (A) and M2 (B). Data represents the mean of triplicate analyses, with error bars showing the standard deviation.

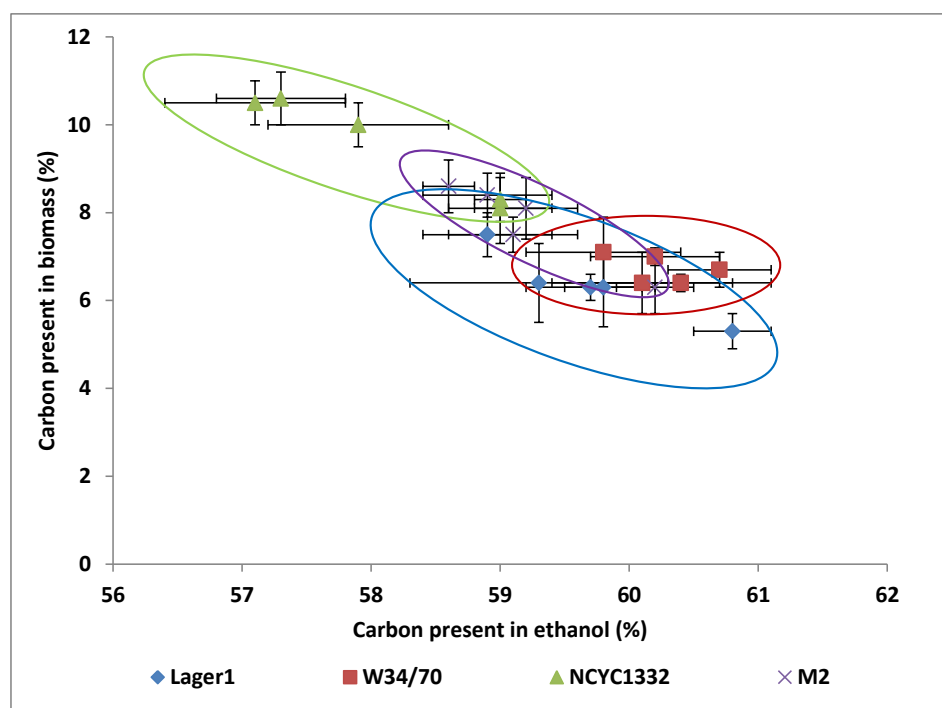


Figure 5.10 Relationship between carbon proportion in the form of biomass and ethanol in different yeasts under the five fermentation conditions. Data represents the mean of triplicate analyses, with error bars showing the standard deviation.

5.3 Discussion

Identification of optimum strategies for directing carbon towards the desired end products is a challenging task owing to the complexity of metabolic networks. Studies of carbon partitioning in microorganisms have led to scientific and industrial breakthroughs in the disciplines of metabolomics (Van Gulik, *et al.*, 2000, Rui, *et al.*, 2010), genetic engineering (Nevoigt, *et al.*, 2002, Underwood, *et al.*, 2002, Cadiere, *et al.*, 2011), and targeted enzyme production (Sauer & Eikmanns, 2005, Grose, *et al.*, 2007). Major example includes the work of Van Gulik, *et al.* (2000) who revealed a number of metabolic bottlenecks within Penicillin fermentation by investigating flux partitioning on different carbon sources, Underwood, *et al.* (2002) who optimized carbon partitioning in an ethanologenic *Escherichia coli* based on a

genetic approach leading to improved xylose fermentation to ethanol, and Rui, *et al.* (2010), who produced a global picture of cellular responses of *E. coli* central carbon metabolic network to superoxide stress. Cadiere, *et al.* (2011) reported an evolutionary engineered *S. cerevisiae* wine yeast with a redirection of carbon flux from glycolysis towards the pentose phosphate pathway.

An example specifically related to beer is the shift of carbon flux towards glycerol at the expense of ethanol formation using a brewing yeast overexpressing gene *GPD1* brewing fermentation, leading to 5.6-fold increase of glycerol production and 18 % reduction of ethanol yield (Nevoigt, *et al.*, 2002). However, apart from this work, the navigation of carbon flow has not been fully explored in the brewing context. The present study not only addresses this, but provides an inexpensive means of predicting fermentation efficiency while also revealing potential strategies for achieving optimized ethanol production. Firstly, the total carbon input was calculated from each carbohydrate group consumed, taking into consideration the net utilisation of wort sugars, especially the notable amounts of residual maltotriose present in the final beers. Secondly, for the first time we provide a comparative estimation of carbon partitioning during HG and VHG fermentations using brewer's wort. These results reveal that the percent carbohydrate conversion to final metabolites including trehalose, glycogen, higher alcohols and esters is not majorly affected by initial wort gravity. At standard gravity the findings reported here are consistent with the assumption of Cutaia (2007) in previous measurements. However, under HG and VHG conditions the absence of a change in carbon utilisation was perhaps surprising, since it was anticipated that increased carbon proportion to cellular protectants such as trehalose and glycerol might be observed. The rationale for this was that at high gravities yeast would require greater concentrations of compounds required to protect or stabilize cell structures. Importantly, the data presented here indicates that even though overall levels of trehalose and glycerol are elevated in HG

and VHG brewing fermentations (Chapter 4), the percentage of carbon directed to these molecules remained consistent. This observation suggests that (I) the concentration of anti-stress agents may not actually be a measure of the yeast stress response under the conditions applied, as the carbon content in the form of these metabolites was basically conserved under both standard and higher gravity conditions; (II) carbon directed towards these products has little impact on ethanol yield. However it should be noted that there are other important considerations which define the success of a fermentation, including fermentation efficiency and yeast ‘fitness’ at high gravities (Stewart, 2009). If metabolites other than ethanol, carbon dioxide and glycerol do not impact significantly on ethanol yield then it may be pertinent to focus on elevating such compounds due to their important functional properties in the yeast. It is possible that only a small increase, negligible in terms of ethanol proportion, would result in significant savings in fermentation time and yeast quality.

The data presented here also indicated that increasing pitching rate may be an effective strategy to shift the carbon flux towards ethanol formation at VHG brewing fermentations. However, it is proposed that in order to achieve an optimized pitching rate, further investigation is needed to compare the effect of cell number on performance using a variety of different yeast strains. Irrespective, the results obtained suggest a trade-off between biomass production and ethanol yield, indicating that ethanol production is not only limited by available wort carbohydrate, but also effected by yeast growth. Previous studies have shown that lipids, such as sterols and unsaturated fatty acids (UFAs), are required for adequate yeast growth (Rosenfeld, *et al.*, 2003) and the major role of oxygen in brewery fermentation is to promote their biosynthesis, which implies that oxygenation may also be significant. Particularly, it is known that the quantity of oxygen supplied at the beginning of fermentation plays a key role in the regulation of the proportion of wort sugars used for the generation of ethanol and new yeast biomass (Boulton & Quain, 2001). Although the oxygen

requirement for growth is likely to be strain-specific (Jakobsen & Thorne, 1980), this topic remains interesting and it is extremely likely that optimization of process parameters such as pitching rate and oxygen levels have the potential to significantly influence carbon investment towards yeast biomass or alcohol.

Despite the relationship between pitching rate, oxygenation, biomass production and ethanol yield, it should be noted that in this study the derived equations employed for determination of yeast mass balance were based on brewer's wort carbohydrate utilisation and measurable outputs. Consequently the 'remaining' carbon proportion was assumed to comprise yeast biomass (yeast biomass 1, Tables 5.2-5.5), which, although a reasonable assumption, was not able to be evaluated accurately. As such, certain carbon biochemical networks may be under-represented, including the pentose phosphate pathway and routes through nitrogen metabolism. Although a direct calculation of carbon content in biomass (yeast biomass 2, Tables 5.2-5.5) yielded broadly comparable data, it should be noted that it was established based on the increase of yeast dry mass and a carbon content of 48 % in a dried wine yeast (Rosen, 1989). Whilst similar composition was also reported in dried baker's yeast (Van Hoek, *et al.*, 1998), in this instance, the occurrence of cell lysis and the carbon composition in individual brewing yeast could be underestimated, especially at HG and VH conditions. A further potential source of discrepancy could be related to the measure of carbon dioxide production; although measurement of weight loss is a simple and economic way to quantify carbon dioxide evolution when analysing multiple small scale fermenters routinely, it is not as accurate as alternative methods. Despite this, the observed ethanol to carbon dioxide ratio was remarkably consistent and certainly within the range representing a theoretical ratio of these compounds. However, with respect to the precise carbon quantification, it should be acknowledged that it may represent a source of either over- or under-estimation, and further investigation would be required to achieve a more accurate framework. Moreover, although

the start and end points of carbon metabolism are of commercial significance (and are the focus of the current study), intermediate compounds such as pyruvate may also provide some useful information regarding carbon flux distribution (Rui, *et al.*, 2010, Quiros, *et al.*, 2013, Soons, *et al.*, 2013). A systematic investigative approach to industrial brewing yeast central carbon metabolism in response to high density wort may yield interesting results and may prove to be a promising topic for the future. In the future, tracer-based techniques could be used as a complementary method to provide greater insight into flux balance analysis as have been applied in other similar studies (Zhang, *et al.*, 2003, Raghevendran, *et al.*, 2004, Blank, *et al.*, 2005, Blank, *et al.*, 2005, Tang, *et al.*, 2009, Christen & Sauer, 2011, Urban, *et al.*, 2011).

5.4 Conclusion

In this chapter an approach was described to evaluate the carbon partitioning of brewing lager and ale strains under a series of lab-scale fermentations using 13 °P, 18 °P and 24 °P brewer's wort. The estimation of total carbon input was calculated to take into consideration the presence of residual sugars under HG and VHG brewing, and was determined from the carbohydrate composition of the original wort and the final beers. Estimation of carbon partitioning revealed that carbon metabolites including trehalose, glycogen, higher alcohols and esters had only minor effects on carbon distribution, whereas yeast biomass acted as a major trade-off with ethanol production, suggesting that the control of cell growth is potentially the most important strategy to navigate carbon into ethanol production, leading to optimized alcohol yield.

**CHAPTER 6: THE EFFECT OF EXTRACELLULAR
OSMOLALITY ON BREWING YEAST CELL
PHYSIOLOGY**

6.1 Introduction

As described in Chapters 1 and 4, the use of High Gravity (HG) and Very High Gravity (VHG) worts has a significant impact on yeast physiology and fermentation characteristics. Many of these effects are related to the metabolic demands created by the wort environment, in particular carbohydrate availability. However, HG and VHG fermentations have also been associated with a number of additional stress factors and in particular elevated osmotic stress, one of the major environmental challenges faced by yeast cells (Stewart, 2009). Osmotic stress can be defined as a challenge occurring when there is an imbalance between intracellular and extracellular osmotic pressure sufficient to initiate an osmotic adjustment within the cell (Cadieux, *et al.*, 1988, Csonka & Hanson, 1991), and can be expressed in the form of osmolality (Section 1.6.2), which can be quantified using an osmometer and commonly used to describe the osmotic pressure of a solution reflecting the milliosmoles of solute per kilogram of solvent (mOsm/kg) (Sweeney & Beuchat, 1993, Erstad, 2003).

It is known that the immediate consequence of exposing yeast cells to high osmotic pressure is a rapid increase in osmotic potential within the cell, resulting from the outflow of water, as well as a decrease in cell volume and turgor (Morris, *et al.*, 1986, Meikle, *et al.*, 1988, Mager & Siderius, 2002). The primary yeast response is triggered by these changes, with extensive gene regulation and the production of a range of protective compounds such as glycerol (Reed, *et al.*, 1987, Meikle, *et al.*, 1988, Hohmann, 2002, Wojda, *et al.*, 2003) and trehalose (Majara, *et al.*, 1996a, Majara, *et al.*, 1996b, Hounsa, *et al.*, 1998). Glycerol is known to act as a compatible solute to counterbalance external pressure and is produced via activation of the High Osmolarity Glycerol (HOG) pathway (Sections 1.4.5.4 and 1.6.3.3), whilst trehalose has been shown to stabilise proteins, internal membrane structures and the plasma membrane (Section 1.4.5.5). This is significant since during osmotic adaptation the plasma membrane functions as a chemi-osmotic barrier, providing the major interface between the organism and

its external environment (Simonin, *et al.*, 2007). Consequently, yeast membrane fluidity has been proposed to be an essential parameter for survival in extreme environments (Learmonth, 2011, Turk, *et al.*, 2011). Under normal conditions, the phospholipid bilayers of biological membranes are thought to be mainly in a liquid-crystalline phase, whereas during stress conditions, phospholipid headgroups are forced close together, leading to the phase transition of phospholipids from liquid-crystalline to gel phase characterised by decreased membrane fluidity (Beney & Gervais, 2001, Simonin, *et al.*, 2008). It has been suggested that such phase transition within cell membrane can be a key step in the induction of cell death as a result of changes in osmotic pressure (Beney & Gervais, 2001, Simonin, *et al.*, 2007, Simonin, *et al.*, 2008, Learmonth, 2011). Interestingly, in addition to membrane effects, previous studies have indicated that DNA damage can be induced by osmotic stress in laboratory yeast strains (Dmitrieva & Burg, 2005, Kultz, 2005, Ribeiro, *et al.*, 2006, Dmitrieva & Burg, 2007, Miermont, *et al.*, 2013). Analysis of chromosomal DNA from yeast cells under hyperosmotic shock has indicated that this can directly result in DNA breakage, leading to the production of fragments of several hundred kilobases in size (Ribeiro, *et al.*, 2006). Furthermore, Miermont, *et al.* (2013) demonstrated that DNA damage may also be related to cell volume reduction caused by severe osmotic stress, which can decrease protein mobility and eventually slow down intracellular signalling and other cellular process, some of which result in DNA damage.

Previous studies of osmotic stress in brewing yeast have typically involved the examination of fermentations using worts of different concentrations, and their effect on product and yeast quality (Panchal & Stewart, 1980, Pratt, *et al.*, 2007, Piddocke, *et al.*, 2009, Sigler, *et al.*, 2009, Yu, *et al.*, 2011, Dekoninck, *et al.*, 2012, Yu, *et al.*, 2012). Current understanding within the industry is that the use of HG and VHG worts results in increased osmotic stress at the beginning of fermentation, due to the high concentration of sugars present (Stewart, 2010,

Puligundla, *et al.*, 2011), after which osmotic stress becomes gradually reduced (Gibson, *et al.*, 2007). However, it is known that the presence of wort sugars is not the only factor involved in determining osmotic potential (Jones & Greenfield, 1987), and the precise osmolality changes in wort media which occur during brewery fermentations, as well as the direct impact of these on brewing yeast, remain mostly unexplored. In particular, there have been no previous reports on the relationship between external osmotic pressure and yeast physiology related to VHG brewing, or the effects of this on brewing yeast cell damage, particularly to DNA and membrane structures. Consequently, the purpose of the current study was to investigate the range of osmolality levels encountered by yeast during standard and higher gravity fermentations, and subsequently to determine the potential effects of osmotic pressure on brewing lager and ale yeast physiology, membrane fluidity and DNA integrity.

6.2 Results

Two lager (Lager1 and W34/70) and two ale (NCYC1332 and M2) brewing yeast strains were examined in this study (Section 2.1). A series of lab-scale fermentations (100 mL wort volume) were conducted using five different experimental conditions as described previously (Sections 2.6 and 4.2). HG (18 °P) and VHG (24 °P) worts were prepared by diluting 25 °P commercial wort (Molson Coors Brewing Company, Burton-on-Trent, UK) to the desired gravity, whilst standard gravity 13 °P commercial wort was also obtained from Molson Coors Brewing Company (UK). In order to mimic industrial wort conditions as closely as possible, 18 °P and 24 °P worts were oxygenated, while 13 °P wort was aerated for a couple of hours prior to pitching as described in Section 2.6.2. As in previous Chapters, 13 °P fermentations were pitched at 1.5×10^7 viable cells/mL and are referred to as 13P15M, while 18 °P fermentations were pitched at both 1.5×10^7 and 1.8×10^7 viable cells/mL, and designated 18P15M and 18P18M, respectively. VHG fermentations at 24 °P were pitched with either 1.5

$\times 10^7$ or 2.4×10^7 viable cells/mL, and are named 24P15M and 24P24M, respectively. The experimental programme was conducted in sequence.

During fermentations, fermenting wort samples were removed at 0, 3, 15, 24, 48, 65, 90 and 120 hours for osmolality determination. In conjunction, individual wort components were also determined (Section 6.2.1). Based on the observed profile of wort osmolality, a series of osmolality levels were then produced artificially using various concentrations of sorbitol (Section 6.2.2), and the cellular response to this stress was determined using both exponential and stationary phase cell populations (Section 6.2.3).

6.2.1 Extracellular osmolality during fermentation and potential contributors

Consideration of the range and extent of osmotic pressure that brewing yeast cells encounter in HG and VHG fermentations is critical since it can not only affect the movement of solutes and water across the cell membrane but may also cause associated cell damage. In order to investigate this, the osmotic pressure of wort (extracellular osmolality) during fermentations was assessed using a micro-osmometer (Section 2.10.1). As expected, the initial wort osmolality was observed to be directly related to wort sugar density, supporting previous publications showing that increasing gravity leads to elevated osmotic pressure (Gibson, *et al.*, 2007, Stewart, 2009). However it was also observed that irrespective of pitching rate, initial gravity and yeast strain applied, the osmolality increased during the course of fermentation (Figures 6.1-6.2). In 13 °P, 18 °P and 24 °P fermentations respectively, extracellular osmolality increased from approximately 700 to 1500 mOsm/kg, from 800 to 1800 mOsm/kg and from 1100 to 2500 mOsm/kg. In each instance a dramatic increase in osmolality was observed to occur between approximately 12-60 hours post-pitching, after which it remained relatively stable at the maximum levels described above (Figures 6.1-6.2). When plotting the osmolality value against specific gravity (Figures 6.3-6.4), the osmolality was observed to

increase with the decreasing wort density throughout each set of fermentations. Furthermore, at specific wort gravity (x-axis), the osmolality level (y-axis) was apparently increased with the starting wort density, irrespective of pitching rate.

Considering the compositional complexity of fermenting wort, the contribution of potential compounds to osmolality was investigated in more details, specifically focusing on the major components of the fermenting wort, i.e. the wort itself, ethanol and glycerol. These were produced by preparing standards as described previously (Section 2.10.2). It should be noted that the concentrations applied were selected to cover the range of individual compounds obtained throughout standard, HG and VHG fermentations, as reported in Chapter 4. It was observed that there was a direct relationship between osmolality and the concentrations of wort ($R^2 = 0.9955$), ethanol ($R^2 = 0.9855$) and glycerol ($R^2 = 0.9985$), respectively (Figures 6.5-6.7). Moreover, it can be seen from Figure 6.5 that wort osmolality became reduced in response to the dilution of an original 24 °P wort, whereas the osmolality of ethanol (Figure 6.6) and glycerol (Figure 6.7) were observed to increase at higher concentrations. Furthermore, it should be noted that the extent to which this occurred was dependent on the type of solute, as indicated by variation in the scale of the y-axis in each instance. Based on the derived equations, comparable osmolality of wort, ethanol and glycerol was demonstrated: the osmolality of 1 % wort (Figure 6.5), 1 g/L ethanol (Figure 6.6) and 1 g/L glycerol (Figure 6.7) are 10.7 mOsm/kg, 19.6 mOsm/kg and 10.2 mOsm/kg, respectively. Given the concentration range of each component observed during fermentations (as indicated in the scale of the x-axis in each figure), it can be concluded that ethanol and wort are the major contributors to extracellular osmolality, whereas glycerol accumulation has a comparatively minor effect during fermentation.

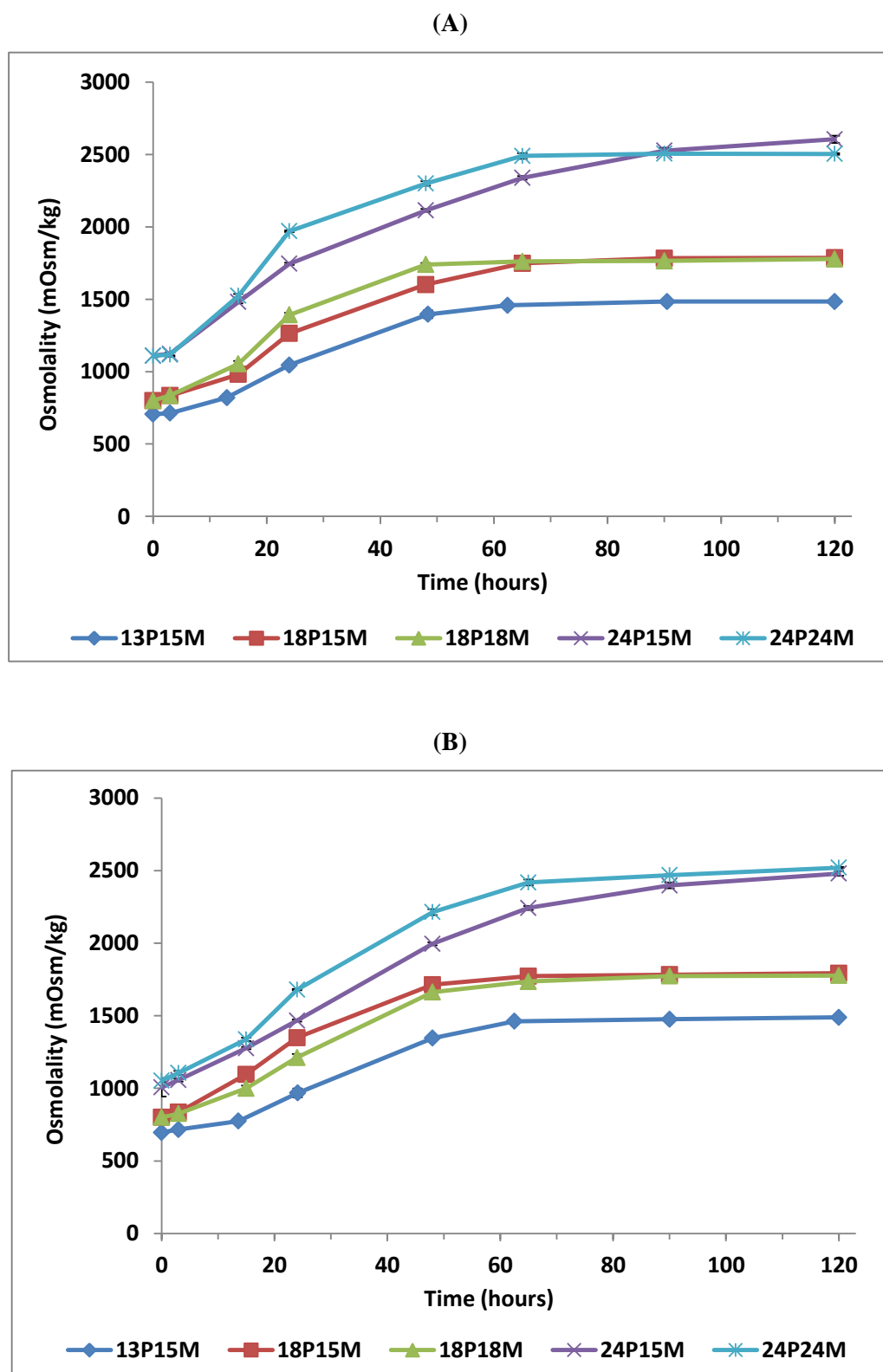


Figure 6.1 Extracellular osmolality during fermentations using lager strains Lager1 (A) and W34/70 (B) as a function of time, assessed using a micro-osmometer. Samples were taken at intervals and yeast was removed through centrifugation. Data points represent the mean of triplicate samples, with error bars showing the standard deviation.

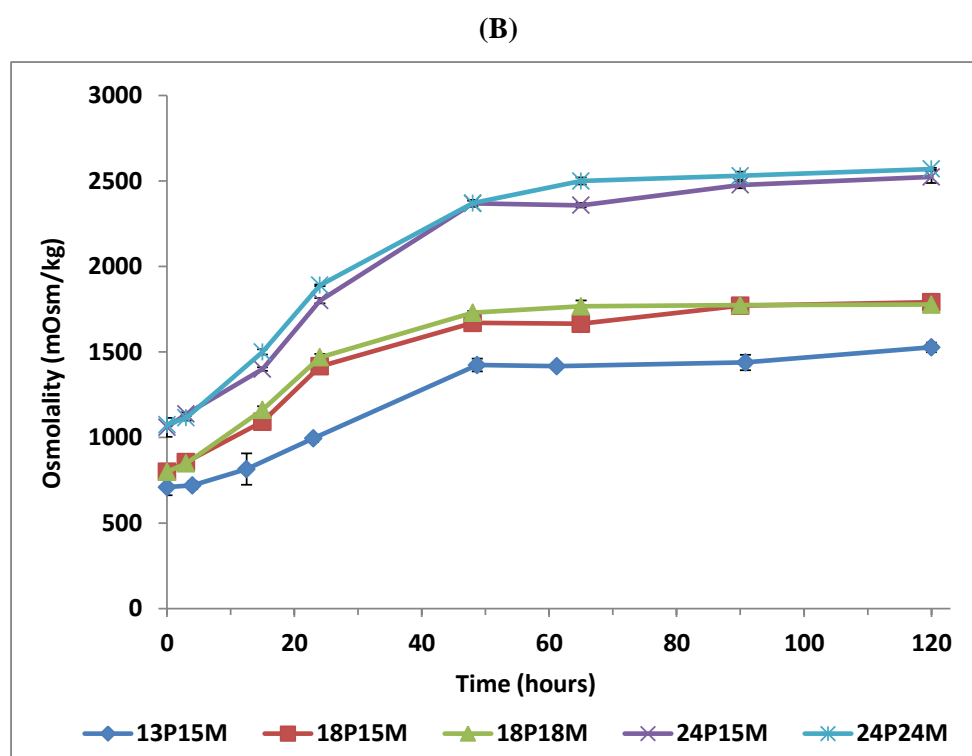
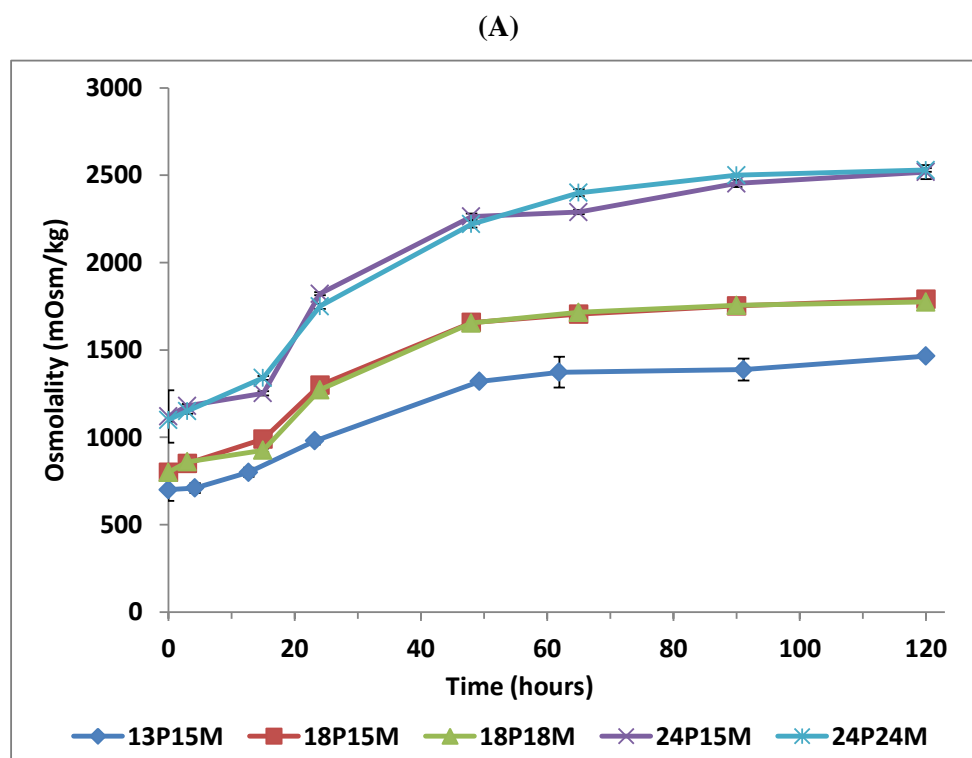


Figure 6.2 Extracellular osmolality during fermentations using ale strains NCYC1332 (A) and M2 (B) as a function of time, assessed using a micro-osmometer. Samples were taken at intervals and yeast was removed through centrifugation. Data points represent the mean of triplicate samples, with error bars showing the standard deviation.

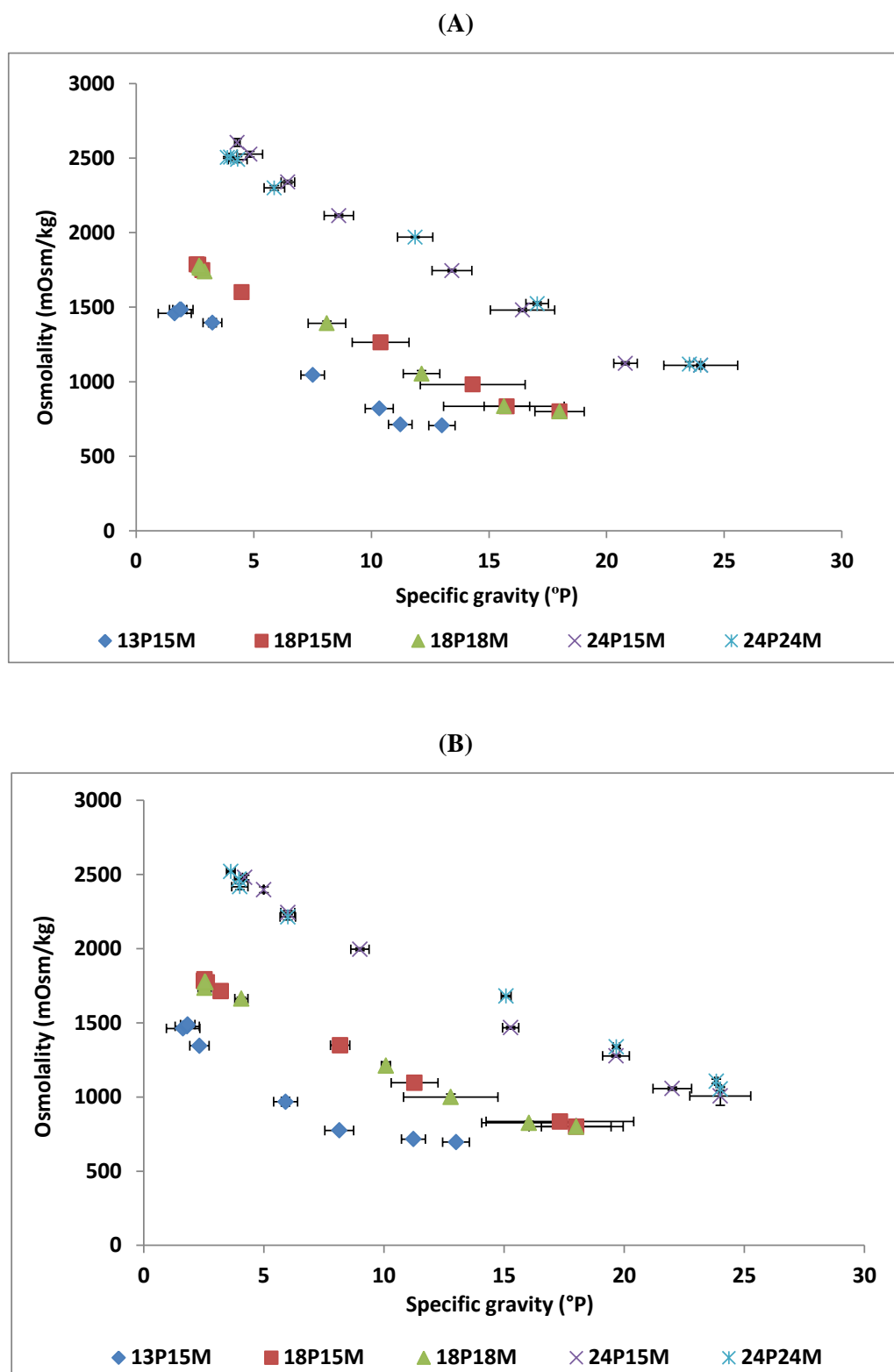


Figure 6.3 Relationship between extracellular osmolality and specific gravity during fermentations using lager strains Lager1 (A) and W34/70 (B). Samples were taken at intervals and yeast was removed through centrifugation. Data points represent the mean of triplicate samples, with error bars showing the standard deviation.

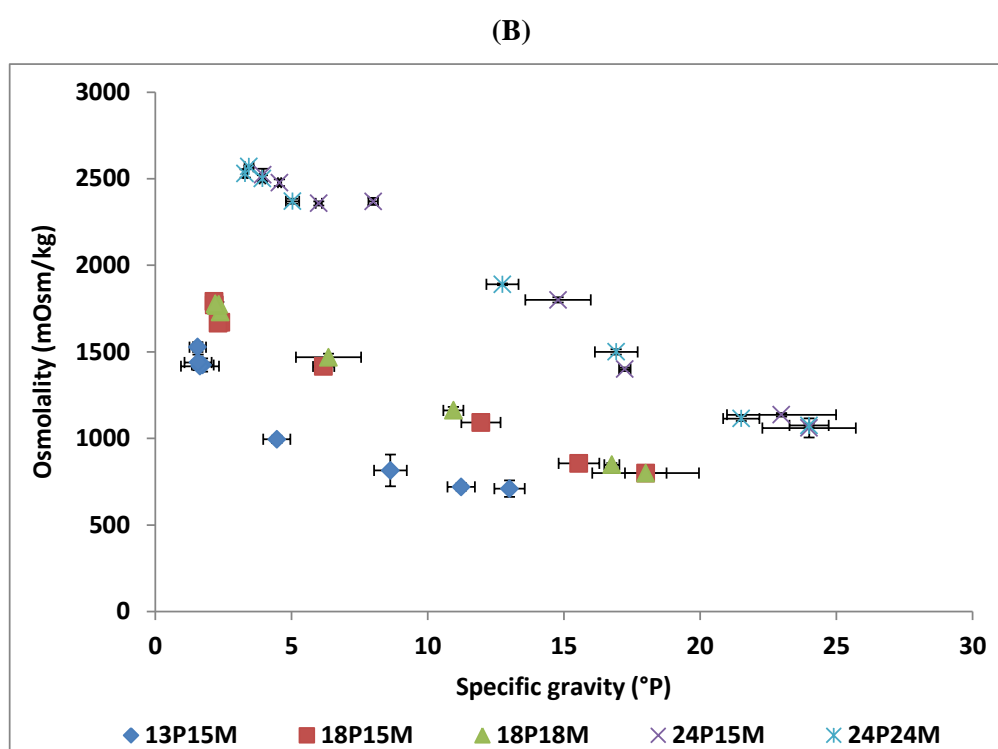
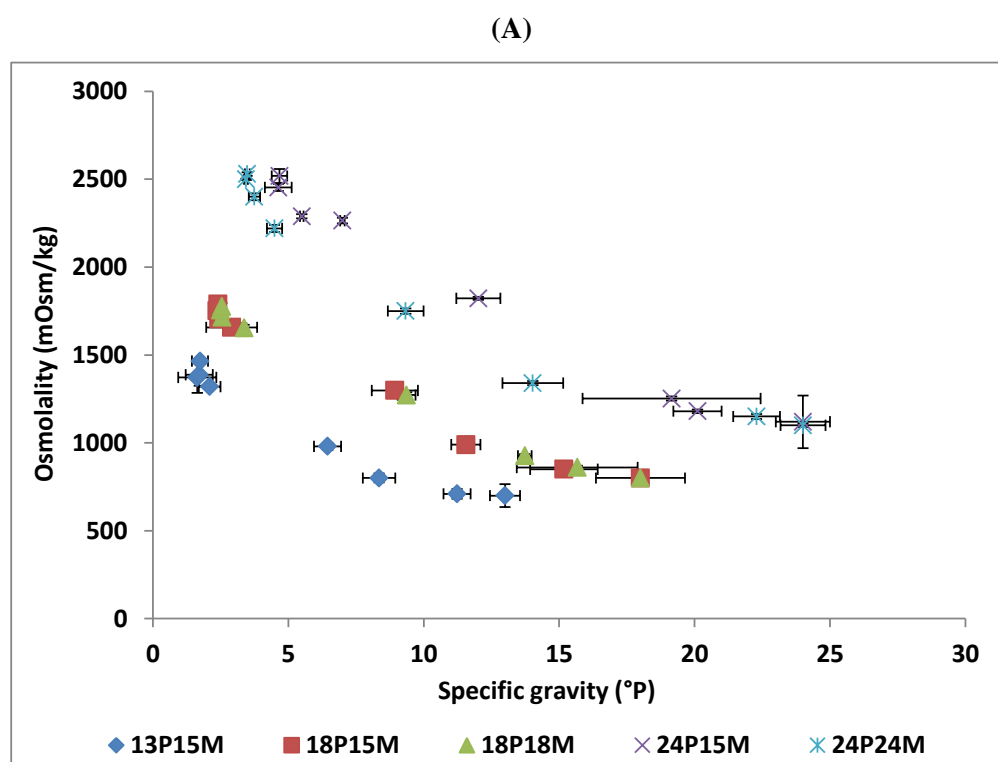


Figure 6.4 Relationship between extracellular osmolality and specific gravity during fermentations using ale strains NCYC1332 (A) and M2 (B). Samples were taken at intervals and yeast was removed through centrifugation. Data points represent the mean of triplicate samples, with error bars showing the standard deviation.

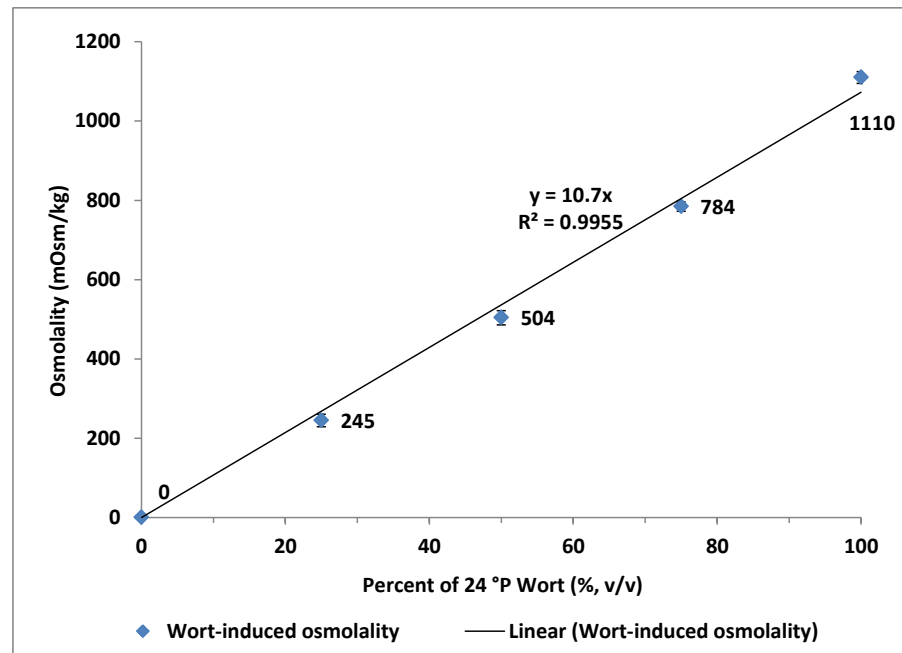


Figure 6.5 Wort osmolality assessed using a micro-osmometer. The osmolality of a series of dilutions of 24 °P brewer’s wort in RO water was determined. Data points represent the mean of triplicate samples, with error bars showing the standard deviation. The mean values and linear equations (y and R^2 values) are shown.

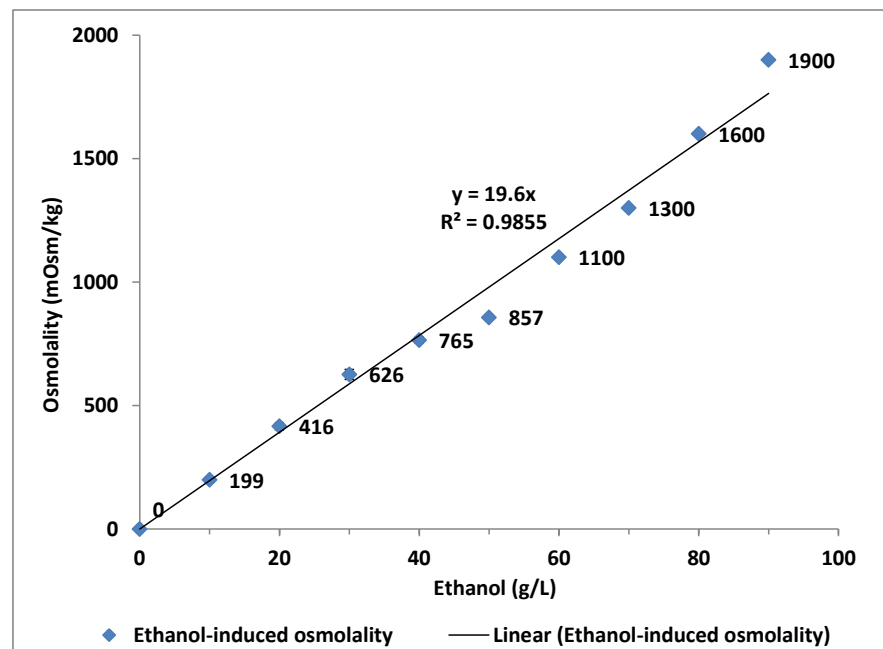


Figure 6.6 Ethanol osmolality assessed using a micro-osmometer. Data points represent the mean of triplicate samples, with error bars showing the standard deviation. The mean values and linear equations (y and R^2 values) are shown.

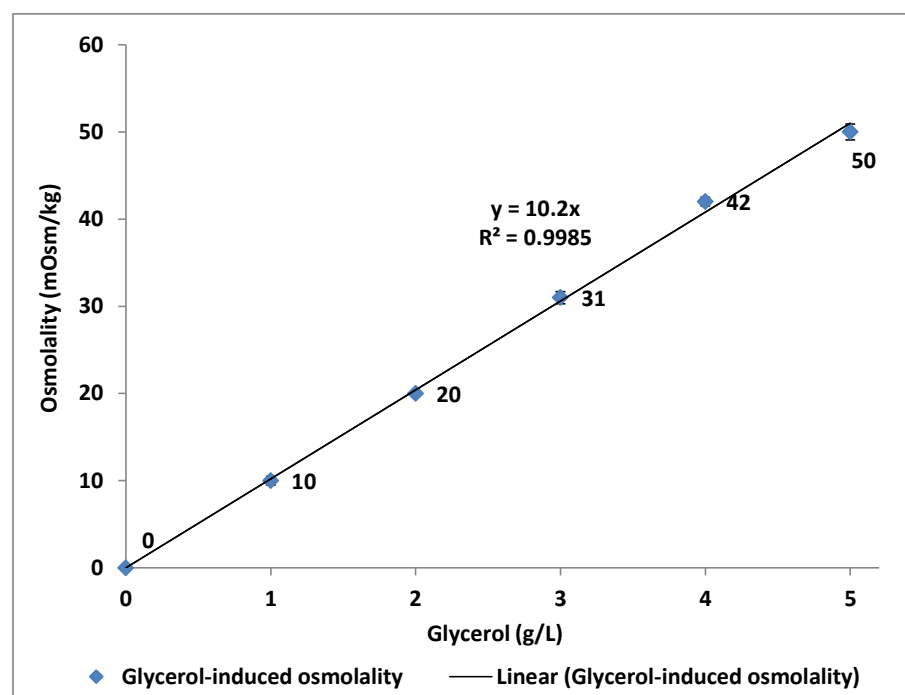


Figure 6.7 Glycerol osmolality assessed using a micro-osmometer. Data points represent the mean of triplicate samples, with error bars showing the standard deviation. The mean values and linear equations (y and R^2 values) are shown.

6.2.2 Development of a sorbitol-induced osmolality system

Sorbitol was selected as an osmotic agent since it does not dissociate into ions in liquids, has no nutritional or toxic effect on yeast cells (Hirasawa, *et al.*, 2006), and has been used previously for the analysis of osmotic stress in a variety of yeast strains (Nass & Rao, 1999, Pratt, *et al.*, 2003, Westfall, *et al.*, 2004, Hirasawa, *et al.*, 2006, Ekberg, *et al.*, 2013, Miermont, *et al.*, 2013). Various concentrations of sorbitol were prepared (Section 2.10.3) with the purpose of mimicking the range of osmolality levels (approximately 650-2500 mOsm/kg) encountered by cells during fermentations as observed previously (Section 6.2.1). As shown in Figure 6.8, sorbitol at various concentrations (10, 20, 30, 40, 50 %, w/v) provided a series of osmolality levels ranging from approximately 560 to 2800 mOsm/kg, alongside a baseline control comprising sterile RO water at 0 mOsm/kg. It should also be

mentioned that, as expected, the extracellular osmolality displayed a good linear relationship ($R^2 = 1$) to the sorbitol concentrations applied. Consequently, this sorbitol-induced osmolality system was used to examine the effect of extracellular osmolality on yeast cell physiology (Section 6.2.3).

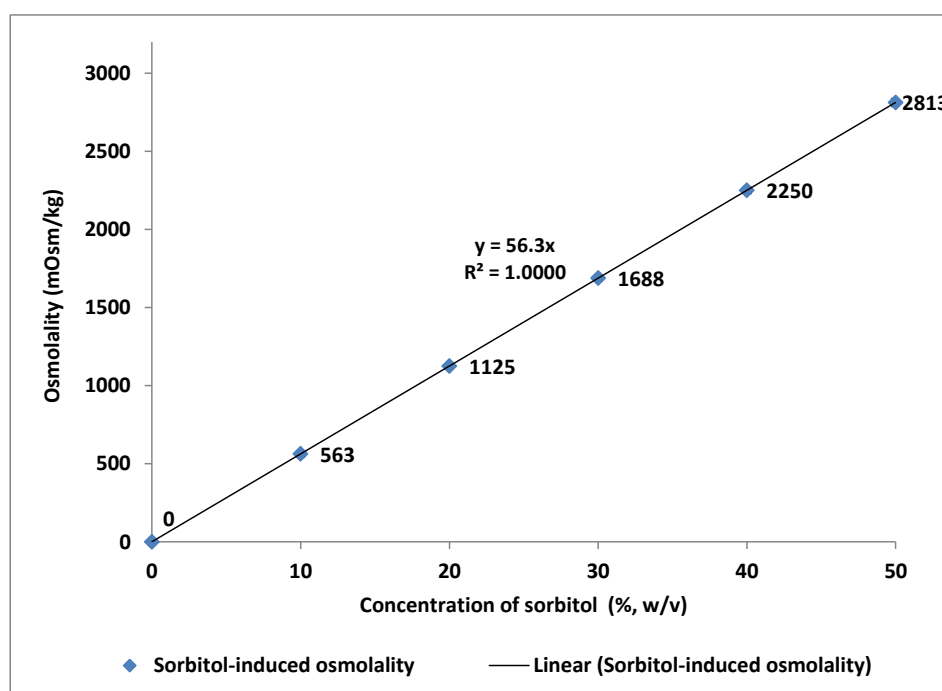


Figure 6.8 Osmolality induced by 0-50 % (w/v) sorbitol, assessed using a micro-osmometer. Data points represent the mean of triplicate samples, with error bars showing the standard deviation. The mean values and linear equations (y and R^2 values) are shown.

6.2.3 The effect of extracellular osmolality on yeast physiology

It is known that the effects of exposing brewing yeast to worts of increased density include a reduction in cell viability (Dekoninck, *et al.*, 2012, Lei, *et al.*, 2012), inhibition of proton efflux rate (Yu, *et al.*, 2011) and the production of stress-related osmo-protectants including

trehalose and glycerol (Piddocke, *et al.*, 2009, Dekoninck, *et al.*, 2012, Yu, *et al.*, 2012). However, in these studies the net effects of osmotic stress alone may have been masked by the combined effects of ethanol toxicity, oxygen availability and nutritional requirements. Consequently, in order to determine the precise effect of extracellular osmolality on cell physiology, a series of osmolality levels were applied to yeast cells. Both exponential and stationary phase cells were examined to reflect the different stages of growth which can be observed during brewing fermentations and to provide an indication of how brewing yeast cultures may react to osmotic pressure at the beginning of fermentation compared to the latter stages. Consequently, the effects of extracellular osmolality on yeast viability and vitality, intracellular trehalose and glycerol, membrane fluidity and genome stability were determined.

6.2.3.1 The effect of extracellular osmolality on yeast viability

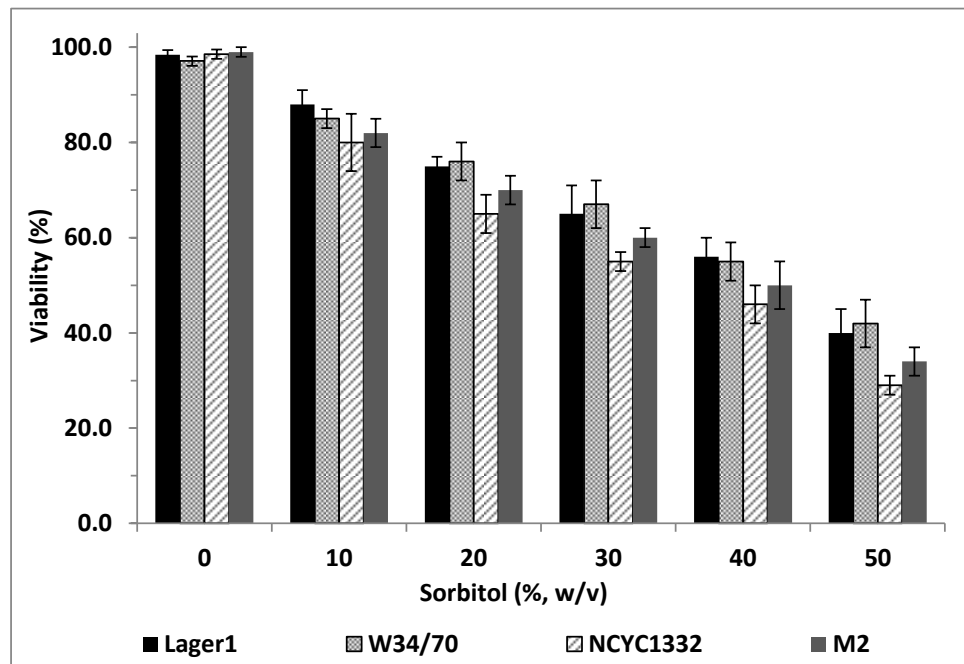
The viability of yeast cells was assessed using brightfield stain methylene blue (Section 2.4.1), as well as fluorescent stains MgANS (1-Anilino-8-naphthalene-sulfonic acid; Section 2.4.2) and oxonol ((bis-(1,3-dibutylbarbituric acid) trimethine oxonol; Dibac₄(3); Section 2.4.3). The application of different staining methods was used to eliminate or reduce the influence of the mode of action of the stain on the potential impact of osmolality. However, although variations in viability were observed when different methods of assessment were used, the application of different staining protocols yielded broadly comparable data.

After 48 hours incubation, cell viability was observed to decrease with increasing external osmolality for each of the four yeast strains, irrespective of growth phase (Figures 6.9-6.10). Furthermore, a general trend was observed whereby stationary phase populations derived from all four yeast strains were more tolerant to osmotic stress than the corresponding exponential phase populations. For example, stationary phase cells from lager yeast populations (Lager1 and W34/70) exhibited a 10-20 % reduction in viability following exposure to 30 % (w/v) sorbitol (Figure 6.10), whereas a 30-40 % reduction in viability was

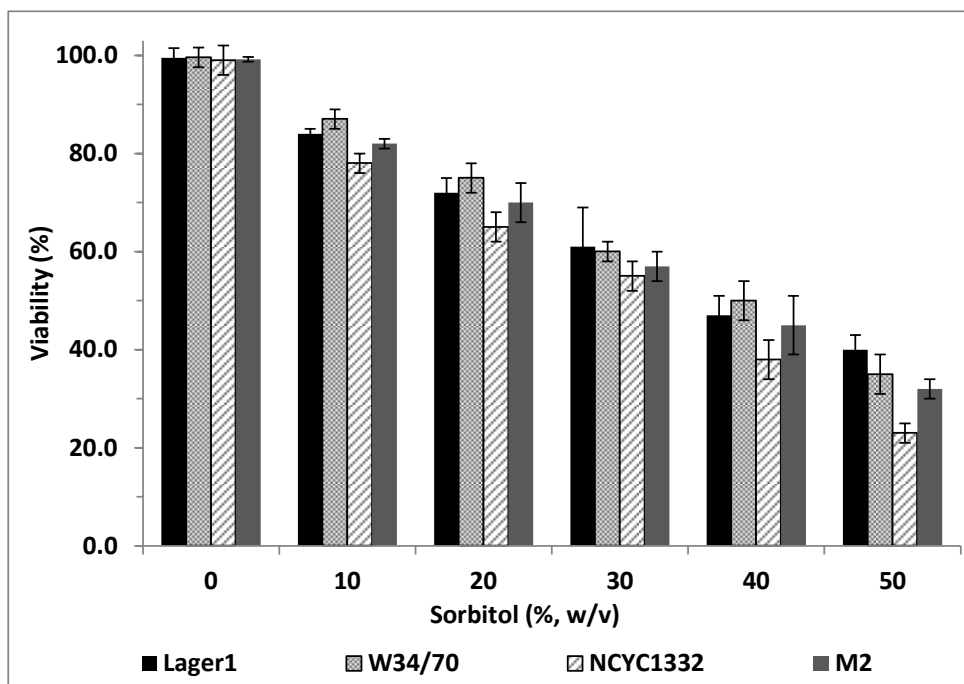
observed for the corresponding exponential phase populations (Figure 6.9). Similarly for the ale strains (NCYC1332 and M2), a 20-30 % and 40-50 % viability loss for stationary (Figure 6.10) and exponential phase populations respectively (Figure 6.9) was detected under the same conditions. These results also indicate that ale yeast strains may be more sensitive to osmotic stress than lager yeasts, since comparable concentrations of sorbitol affected ale yeast populations to a greater extent than lager populations.

In addition to potential variation due to yeast type, it was noticed that each strain displayed a unique pattern of tolerance as a result of the change in extracellular osmolality. Specifically, the ale strain NCYC1332 was observed to be particularly osmo-sensitive. Within the range of sorbitol concentrations and osmolality utilised (0-50 % [w/v], 560-2800 mOsm/kg), stationary phase populations derived from this strain exhibited a viability reduction of approximately 50 % following exposure to 50 % (w/v) sorbitol (Figure 6.10), and a more marked viability loss (ca. 40 %) was observed for the corresponding exponential phase populations in the presence of 40 % (w/v) sorbitol (Figure 6.9). Given that strain NCYC1332 appeared to be sensitive to osmotic shock, this strain was selected for further analysis, along with lager yeast W34/70 as a representative lager type strain, in order to investigate specific osmotic-derived effects on yeast populations, as described in subsequent sections.

(A)



(B)



(C)

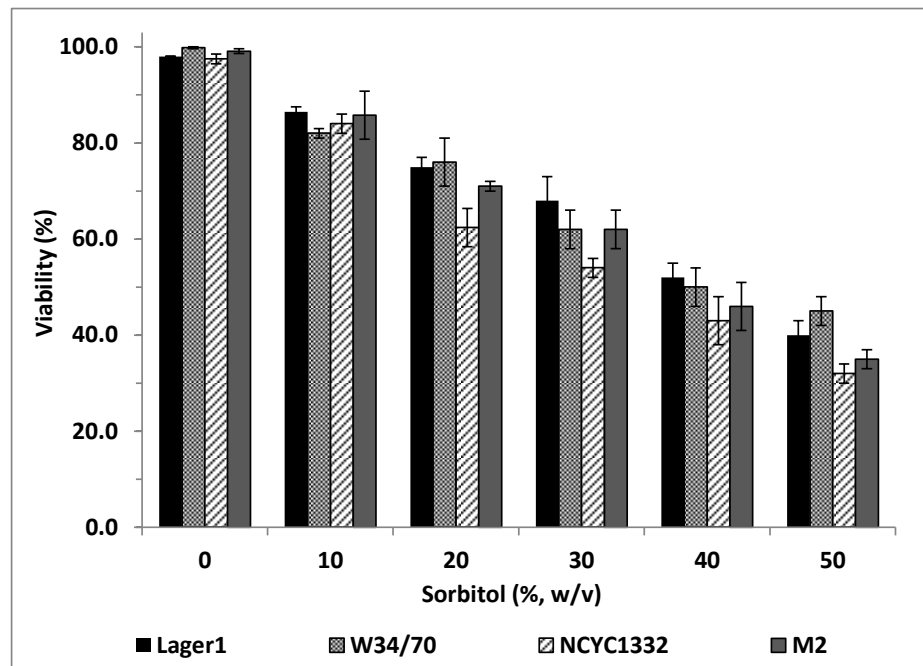
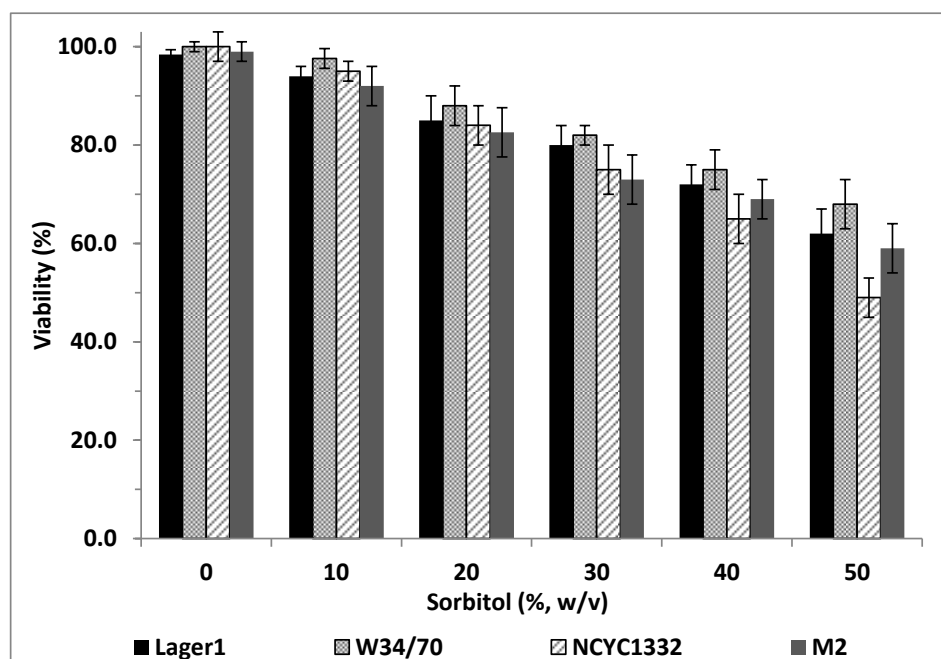


Figure 6.9 Viability of exponentially-growing yeast cells following exposure to a series of osmolality levels induced by sorbitol over 48 hours. Yeast viability was assessed using methylene blue (A), MgANS (B) and oxonol (C). Error bars represent standard deviation from the mean values of triplicate samples.

(A)



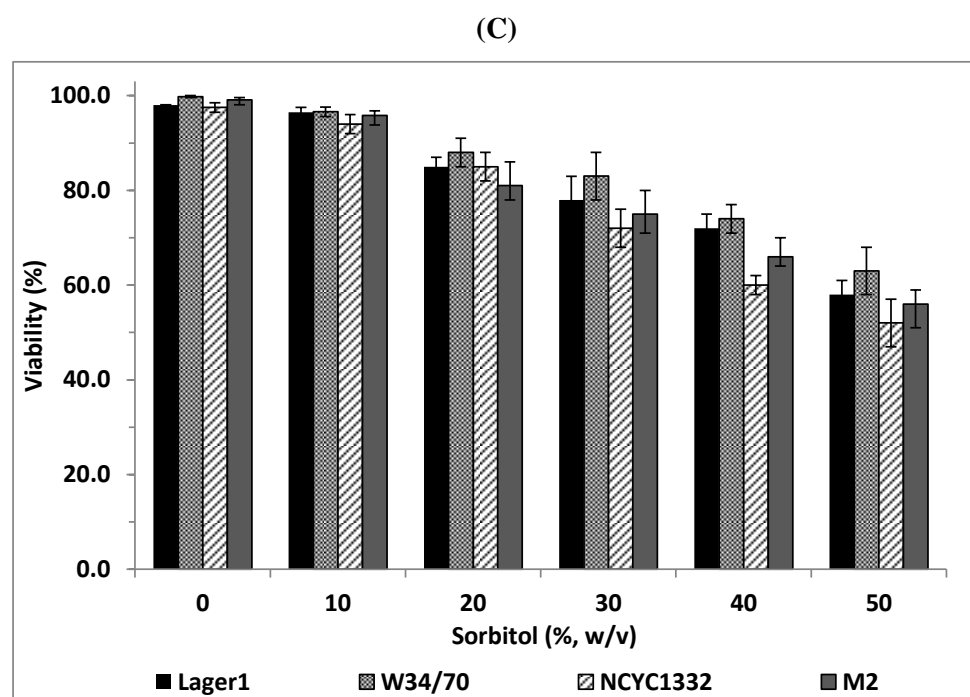
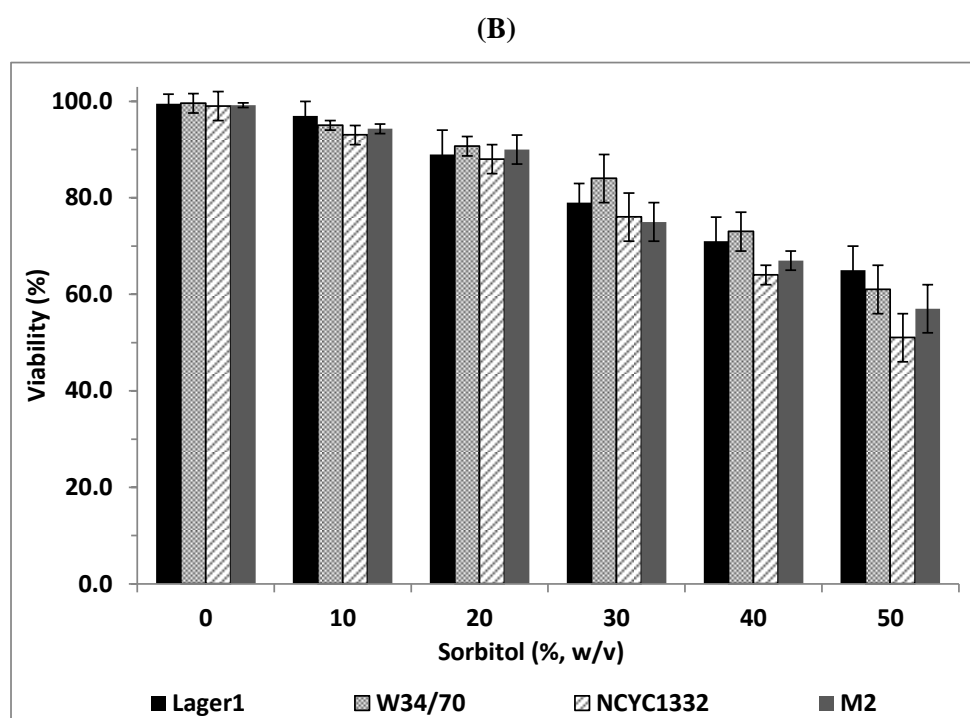


Figure 6.10 Viability of stationary phase yeast cells following exposure to a series of osmolality levels induced by sorbitol over 48 hours. Yeast viability was assessed using methylene blue (A), MgANS (B) and oxonol (C). Error bars represent standard deviation from the mean values of triplicate samples.

6.2.3.2 The effect of extracellular osmolality on yeast vitality

‘High quality’ brewing yeast populations are not only defined by cell viability, but by their activity, or vitality, which is a measure of their capacity to perform essential cellular functions. The acidification power test (Section 2.11), based on proton efflux in response to sugar utilisation, can be used as a measure of yeast activity, allowing fermentation performance of a brewing yeast culture to be predicted (Siddique & Smart, 2000, Sigler, *et al.*, 2006, Gabriel, *et al.*, 2008, Li, *et al.*, 2014). In the current study, this assay was performed to indicate net glucose induced proton efflux (GIPE), pre- (control) and post- exposure to a series of extracellular osmolality levels as a measure of population vitality, where high GIPE values indicate good vitality. Exponential and stationary phase cells of the lager strain W34/70 and the ale strain NCYC1332 were examined as previously described.

In general, GIPE (vitality) values were observed to decrease with increasing osmolality, regardless of yeast strain and growth phase applied (Figure 6.11). Although the data obtained were similar for both yeast strains prior to and after treatment with 0 % (w/v) sorbitol (control), the ale strain NCYC1332 showed a lower ($P < 0.05$) GIPE than the lager strain W34/70 following exposure to both 10 and 20 % (w/v) sorbitol (corresponding to 563 and 1125 mOsm/kg), respectively. Nevertheless, this trend was not maintained in the presence of 30, 40 and 50 % (w/v) sorbitol, where no significant difference between the two strains was observed. This was interesting since it indicated that although strain NCYC1332 appears to be less tolerant to high osmolality (Section 6.2.3.1), population vitality was not proportionally influenced by osmotic pressure. Moreover, whilst GIPE values for exponential and stationary phase populations for each strain were similar under 0-30 % (w/v) sorbitol, it was noticed that they were higher ($P < 0.05$) in stationary phase cells (Figure 6.11B) than in the corresponding exponentially growing populations (Figure 6.11A) in the presence of 40 and 50 % (w/v) sorbitol, indicating that stationary phase cells were able to tolerate high osmolality conditions more readily than exponential cells.

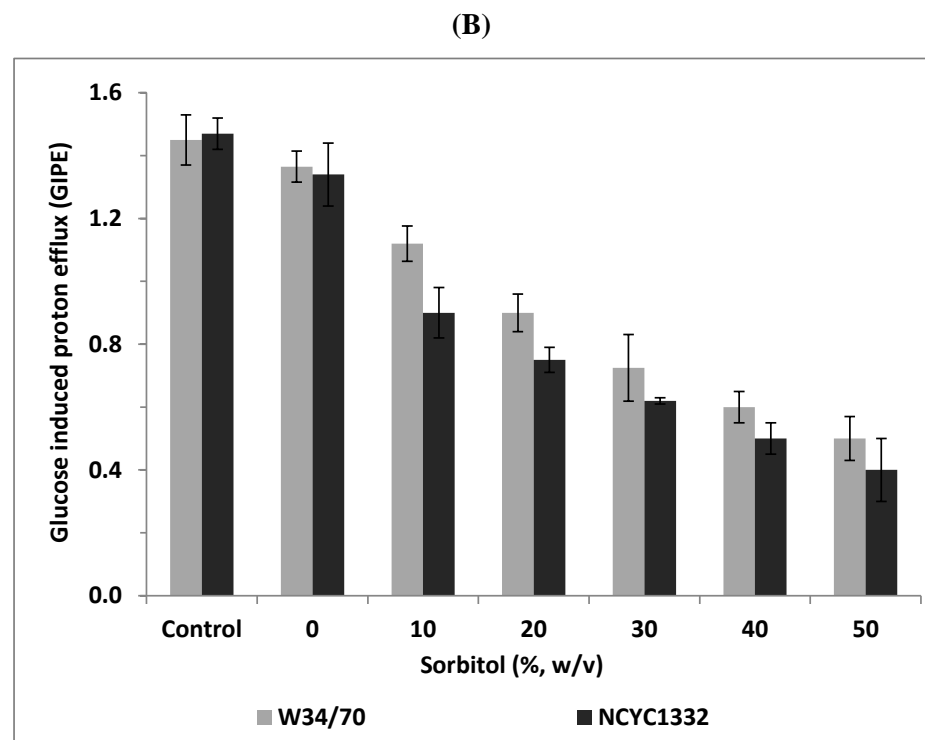
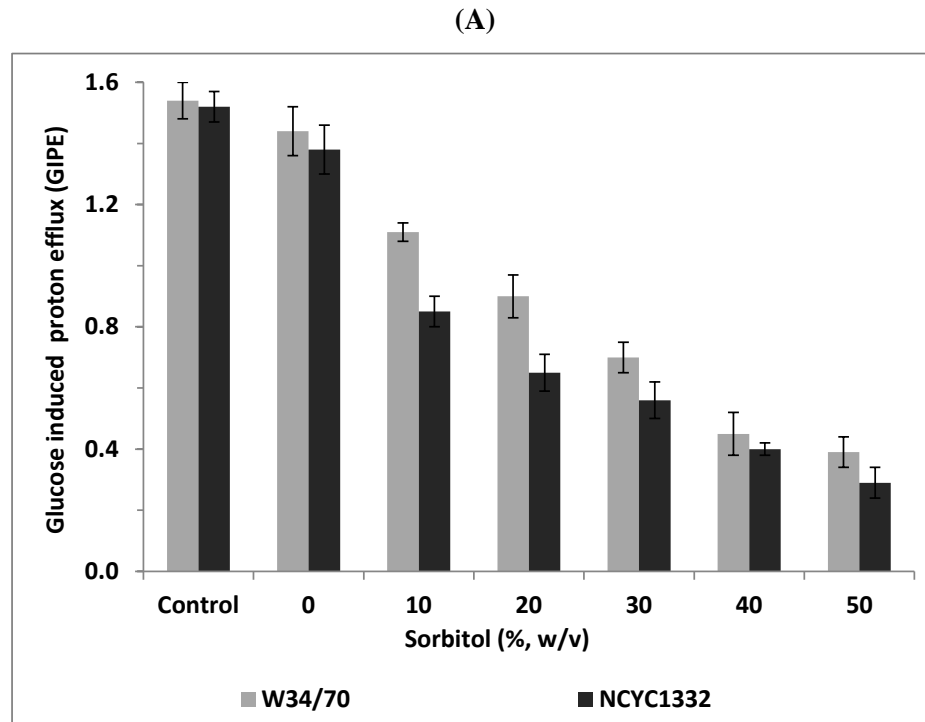


Figure 6.11 Glucose induced proton efflux (GIPE) of W34/70 and NCYC1332 yeast cells prior to (control) and post exposure to a series of osmolality levels induced by sorbitol over 48 hours. Exponential (A) and stationary phase (B) cells were examined using acidification power test. Values represent the mean of triplicate samples and the standard deviation is indicated by error bars.

6.2.3.3 The effect of extracellular osmolality on intracellular trehalose and glycerol

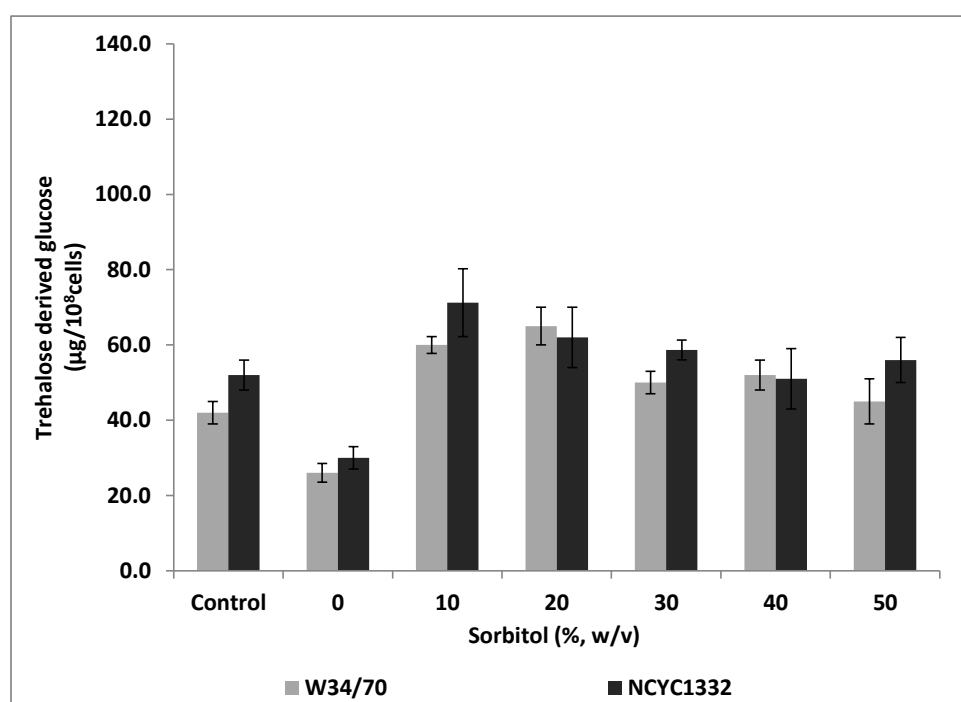
Trehalose and glycerol have been regarded to be important stress protectants in brewing yeast used for HG and VHG fermentations (Majara, *et al.*, 1996a, Piddocke, *et al.*, 2009, Dekoninck, *et al.*, 2012, Yu, *et al.*, 2012), however the precise relationship between their cellular production and the external osmolality has not been fully elucidated. To investigate the net effect of extracellular osmolality on accumulation of these two compounds in brewing yeast, concentrations of trehalose and glycerol were determined in cells pre- (control) and post exposure to a series of extracellular osmolality induced by sorbitol over a 48 hours period.

A marked decrease in trehalose was observed in cells following exposure to 0 % (w/v) sorbitol (control), irrespective of growth phase and yeast strain applied (Figure 6.12), indicating possible starvation-induced utilisation of trehalose. However, analysis of trehalose in response to external osmotic pressure indicated that production was elevated, albeit similar within the range of sorbitol concentrations utilised. Interestingly, no significant difference was observed between the two strains analysed, irrespective of growth phase. However, it was noticed that stationary phase cells produced higher ($p < 0.05$) amounts of trehalose than the corresponding exponentially growing populations in response to osmotic pressure.

Analysis of samples for glycerol production indicated that there was a dramatic decrease in the concentration of this molecule following exposure to RO water (0 % [w/v] sorbitol; control), regardless of growth phase (Figure 6.13). This could be explained by the opening of Fps1p driven glycerol channels (Luyten, *et al.*, 1995) due to a hypo-osmotic shock and the cells requirement to export glycerol to prevent bursting (Luyten, *et al.*, 1995, Tamas, *et al.*, 1999). However, irrespective of this, glycerol production became elevated as the concentration of sorbitol was increased from 10 to 50 % (w/v) (corresponding to 563 to 2813

mOsm/kg), with a marked increase in the presence of 20 % (w/v) sorbitol in the lager strain W34/70. This indicates that osmolality level of 1125 mOsm/kg may act as a major trigger in the activation of the HOG pathway, leading to accumulation of cellular glycerol. Moreover, the lager strain W34/70 produced higher amount of glycerol than the ale strain NCYC1332 within the range of sorbitol concentrations utilized (10-50 %, w/v), irrespective of growth phase applied, corresponding to previous observations indicating that this ale strain is particularly sensitive to osmotically challenging environments.

(A)



(B)

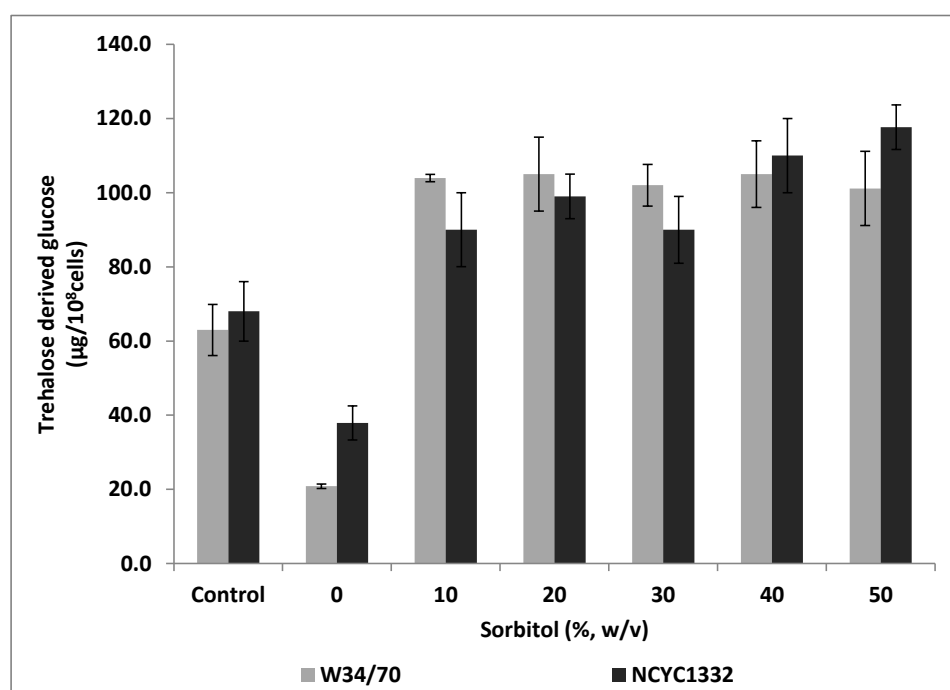


Figure 6.12 Concentrations of trehalose in W34/70 and NCYC1332 yeast cells prior to (control) and post exposure to a series of osmolality levels induced by sorbitol over 48 hours. Exponential (A) and stationary phase (B) cells were examined. Values represent the mean of triplicate samples and the standard deviation is indicated by error bars.

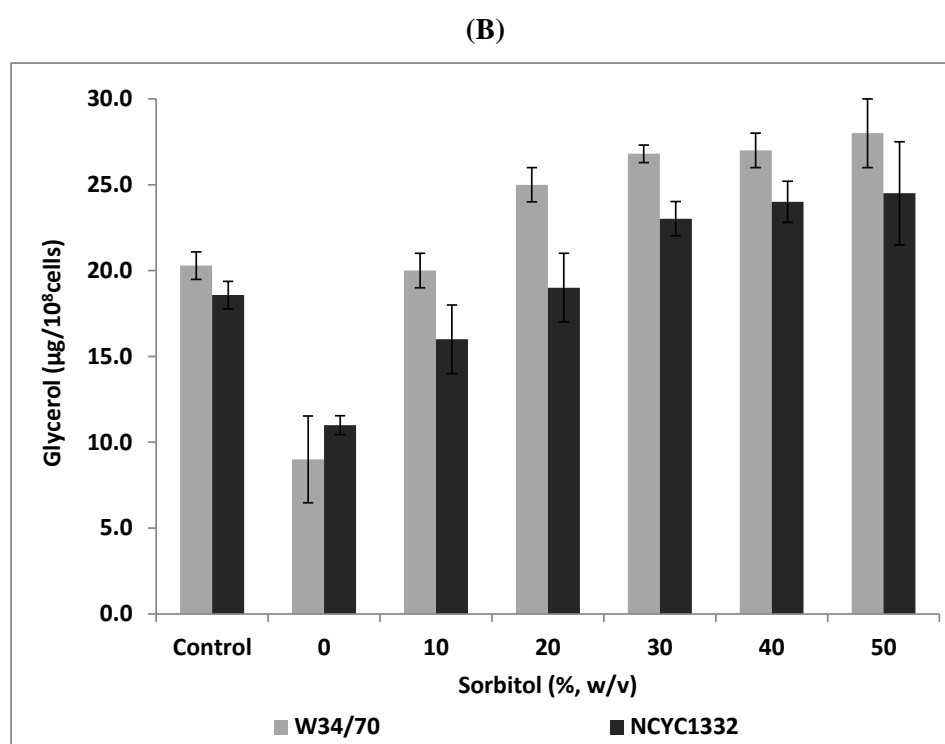
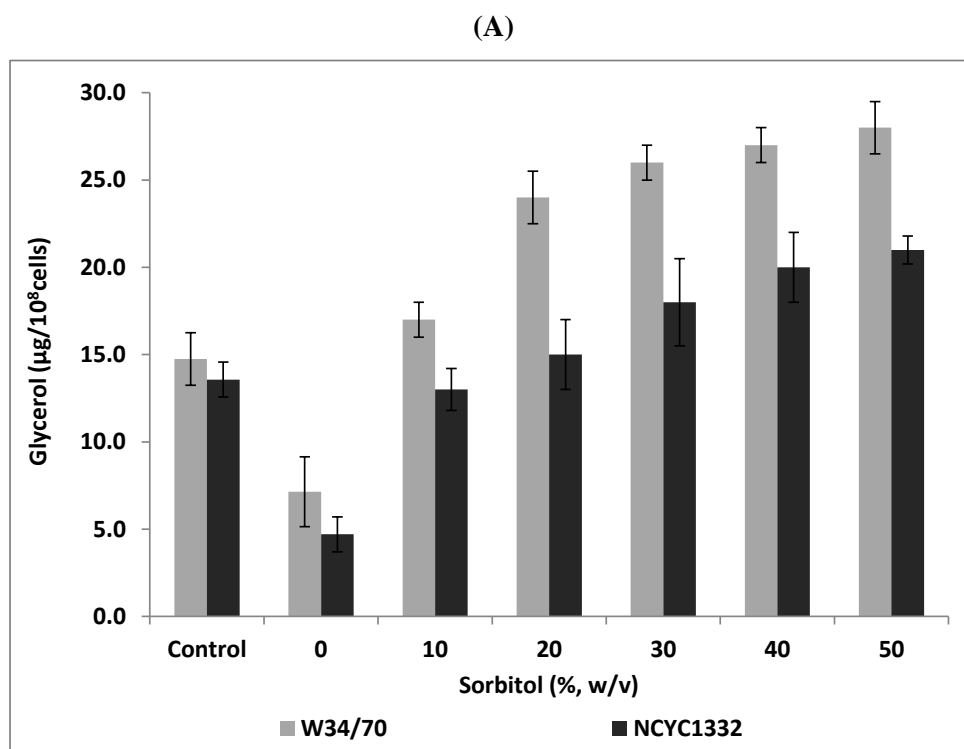


Figure 6.13 Concentrations of glycerol in W34/70 and NCYC1332 yeast cells prior to (control) and post exposure to a series of sorbitol-induced osmolality over 48 hour. Exponential (A) and stationary phase (B) cells were examined. Values represent the mean of triplicate samples and the standard deviation is indicated by error bars.

6.2.3.4 The effect of extracellular osmolality on yeast membrane fluidity

The plasma membrane is believed to be one of the primary targets of physical stress in yeast since it forms a semi-permeable barrier between the organism and its external environment. The fluidity of the membrane can be affected by various types of stress, leading to cellular damage and cell death (Beney & Gervais, 2001, Learmonth & Gratton, 2002, Turk, *et al.*, 2011). However, no analysis of the effect of external osmolality on this cell parameter in brewing yeast has previously been performed.

To determine the relationship between extracellular osmolality and membrane fluidity, a study of yeast cells prior to (control) and post exposure to a series of extracellular osmolality induced by sorbitol was conducted. Fluidity was measured by fluorescence spectroscopy using laurdan Generalized Polarization (GP) (Section 2.12) as an index of membrane fluidity (Learmonth & Gratton, 2002, Walker, *et al.*, 2006, Sanchez, *et al.*, 2012). This was calculated from relative fluorescence intensities at wavelengths representing the gel (440 nm) and liquid-crystalline (490 nm) phases, where low GP levels indicate high membrane fluidity and vice versa. For each yeast strain, the GP value (Figure 6.14) increased (indicating a decrease in membrane fluidity) following exposure to the negative control conditions (0 % [w/v] sorbitol), regardless of yeast strain or growth phase. It should be noted that this baseline control is perhaps misleading since it represents a hypotonic environment, however it was interesting to note that yeast cells exposed to mild osmotic pressure (10 % [w/v] sorbitol) exhibited a similar GP value to the control sample. In contrast, at higher concentrations a gradual increase in GP was observed until a stable value was obtained under elevated osmotic pressure (40 and 50 % [w/v] sorbitol). In addition, GP values obtained from analysis of exponential phase cells were consistently higher (Figure 6.14A) than those of the corresponding stationary phase populations (Figure 6.14B) following exposure to 20-50 %

(w/v) sorbitol for the two strains studied, indicating greater membrane fluidity in stationary phase cells. However, despite this trend, when each strain was compared, stationary phase cells from the lager strain W34/70 displayed higher ($P < 0.05$) GP levels than those of the ale strain NCYC1332 in the presence of 10 and 20 % (w/v) sorbitol, although at higher sorbitol concentrations there was no significant difference between the two yeasts (Figure 6.14B).

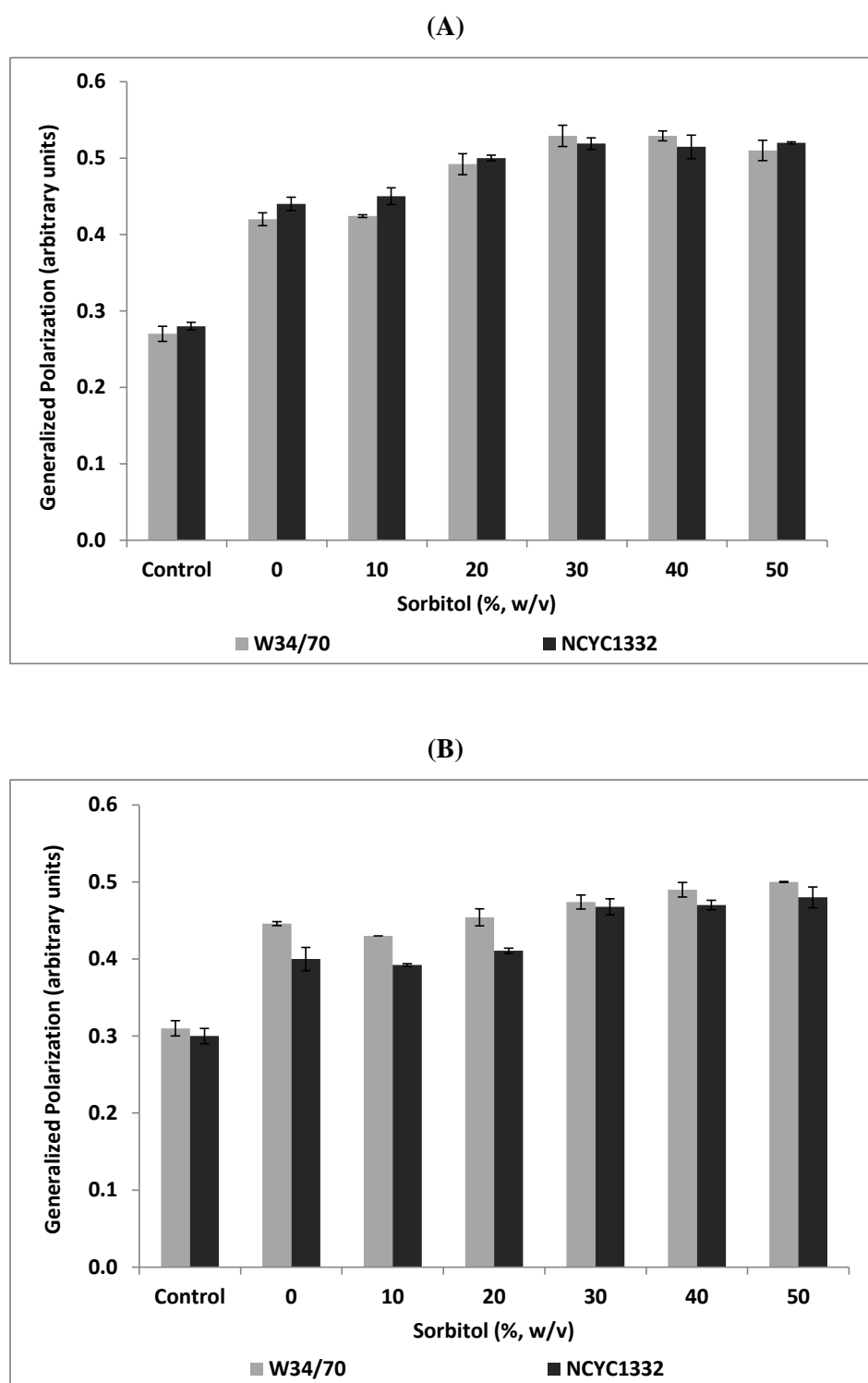


Figure 6.14 Membrane fluidity of yeast strains W34/70 and NCYC1332 prior to (control) and post exposure to a series of osmolality levels induced by sorbitol over 48 hours, as indicated by Generalized Polarization (GP). Exponential (A) and stationary phase (B) cells were examined using fluorescence spectroscopy in conjunction with laurdan staining. Low GP values indicate high membrane fluidity. Data represents the mean of triplicate samples and the standard deviation is indicated by error bars.

6.2.3.5 The effect of extracellular osmolality on genomic stability of inter-delta regions

In order to further investigate potential damage caused by changes in extracellular osmolality, DNA fingerprinting was performed to analyse DNA inter-delta regions (Legras & Karst, 2003) in the lager (W34/70) and ale (NCYC1332) brewing yeast strains. Exponential and stationary phase cells were exposed to a range of extracellular osmolality levels induced by sorbitol and subsequently DNA fingerprinting was conducted by analysis of yeast inter-delta regions using PCR (Section 2.13.4). Examination of inter-delta DNA profiles obtained from fresh yeast cells indicated that each strain displayed a unique DNA fingerprint (Figures 6.15-6.18, lanes 10-11; comparison between individual figures). However, following exposure to a range of osmolality levels (0-50 % [w/v] sorbitol), DNA patterns were unaffected and a genetically identical profile was observed within each strain, irrespective of growth phase (exponential or stationary phase cells) (Figures 6.15-6.18).

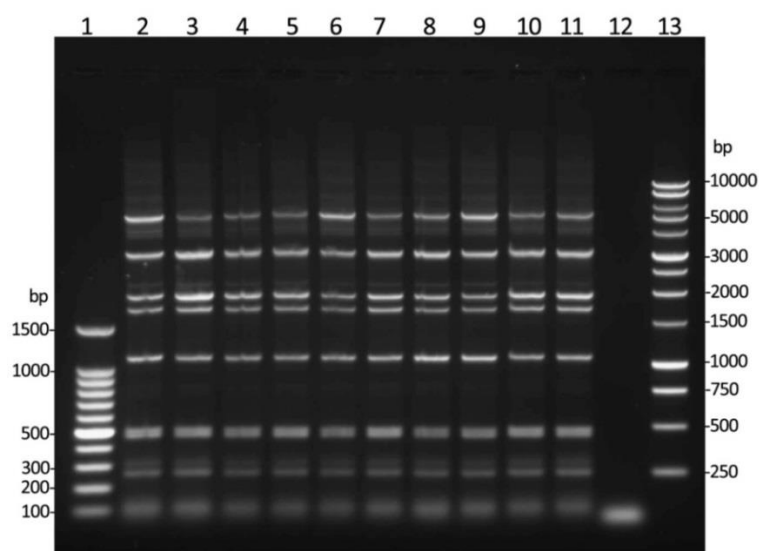


Figure 6.15 Analysis of DNA inter-delta regions derived from exponential phase lager yeast W34/70 by DNA fingerprinting prior to (control) and post exposure to a series of osmolality levels induced by sorbitol over 48 hours. Lane 1: 100bp ladder; lane 2-3: 0 % sorbitol; lane 4-5: 10 % sorbitol; lane 6-7: 30 % sorbitol; lane 8-9: 50 % sorbitol; lane 10-11: control; lane 12: blank (pure water); lane 13: 1Kb marker.

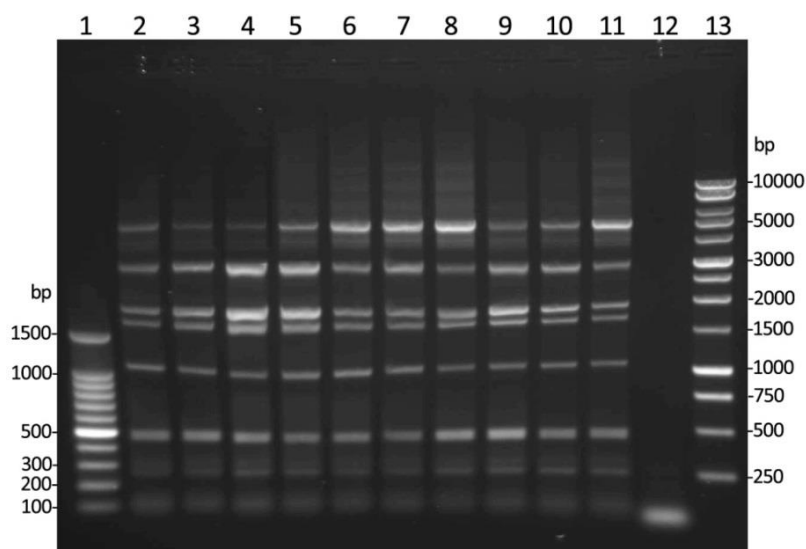


Figure 6.16 Analysis of DNA inter-delta regions derived from stationary phase lager yeast W34/70 by DNA fingerprinting prior to (control) and post exposure to a series of osmolality levels induced by sorbitol over 48 hours. Lane 1: 100bp ladder; lane 2-3: 0 % sorbitol; lane 4-5: 10 % sorbitol; lane 6-7: 30 % sorbitol; lane 8-9: 50 % sorbitol; lane 10-11: control; ; lane 12: blank (pure water); lane 13: 1Kb marker.

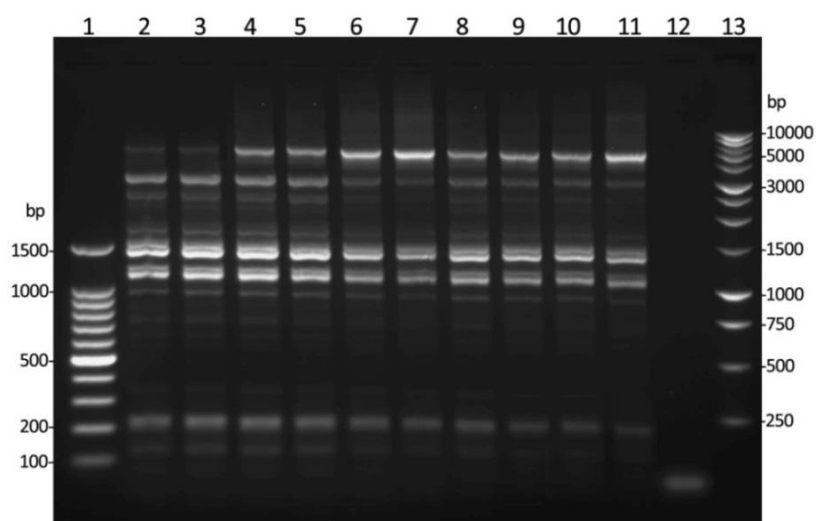


Figure 6.17 Analysis of DNA inter-delta regions derived from exponential phase ale yeast NCYC1332 by DNA fingerprinting prior to (control) and after exposure to a series of osmolality levels induced by sorbitol over 48 hours. Lane 1: 100bp ladder; lane 2-3: 0 % sorbitol; lane 4-5: 10 % sorbitol; lane 6-7: 30 % sorbitol; lane 8-9: 50 % sorbitol; lane 10-11: control; lane 12: blank (pure water); lane 13: 1Kb marker.

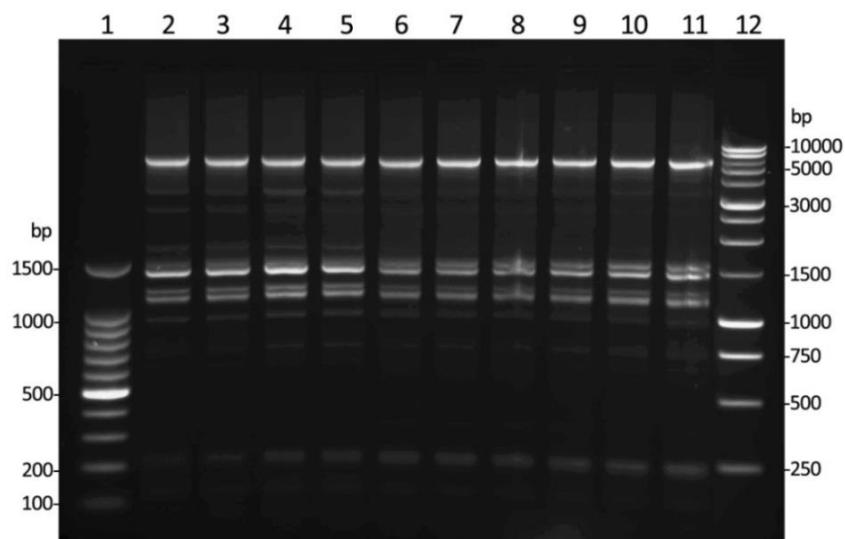


Figure 6.18 Analysis of DNA inter-delta regions derived from stationary phase ale yeast NCYC1332 by DNA fingerprinting prior to (control) and post exposure to a series of osmolality levels induced by sorbitol over 48 hours. Lane 1: 100bp ladder; lane 2-3: 0 % sorbitol; lane 4-5: 10 % sorbitol; lane 6-7: 30 % sorbitol; lane 8-9: 50 % sorbitol; lane 10-11: control; lane 12: 1Kb marker.

6.3 Discussion

In this study the osmolality of fermenting media was determined and used to investigate the effect of extracellular osmotic pressure on yeast cell physiology. It was observed that increasing wort density resulted in elevated external osmolality at the beginning of fermentations, as expected. However, once sugars began to be utilized, the extracellular osmolality was observed to increase throughout fermentation, irrespective of original wort gravity or the yeast strain applied. This observation was perhaps surprising given that sugar concentration, believed to be a major contribution to environmental osmotic pressure, is reduced during fermentations.

The significance of this finding needs to be emphasised since it suggested that the osmotic stress increases during fermentations, probably contradictory from a brewer's viewpoint and from the current 'state of the art' in broader fermentations (Gibson, *et al.*, 2007). It is suggested that this increase can therefore be attributed to the result of yeast metabolism and the changing complexity of the medium, including the constitution of the wort and the increase in both ethanol and glycerol. Indeed, it was demonstrated that each of these components exerts an osmotic pressure, with the largest impact arising from the presence of ethanol. Our data indicated that ethanol-induced osmotic pressure may play an important role in brewing fermentation, and that this effect may be exacerbated by the use of HG and VHG worts. This finding is in accordance with Jones and Greenfield (1987), who suggested that the non-specific inhibitory effect of ethanol can be characterized by reduced water activity (i.e. increased solution osmolality). The same authors also provided evidence to suggest that the inhibitory effects of ethanol were significantly greater than substrate sugars for one of the strains in their study, again providing partial support to the data presented here. The significant contribution of ethanol to osmolality observed in this study provides a springboard for future work on the implication of protracted osmotic pressure for both brewery and other yeast-based fermentation systems (e.g. wine, biofuels).

6.3.1 The impact of extracellular osmolality on cell health

In order to investigate the effect of extracellular osmolality on yeast cellular response, exponential and stationary phase cells were exposed to a range of osmolality levels, induced by sorbitol. This was designed to subject brewing yeast cells at different stages of growth to the range of external osmolality levels that yeast would typically encounter during fermentation, without additional nutritional or toxic effects.

Cell viability is one of the most basic criteria which can be used to evaluate the health status of a yeast cell population, and this was assessed using different stains. Methylene blue is the most widespread dye used for viability assessment within the brewing industry, and its mode of action is based on its ability to stain dead cells blue by penetrating the cell membrane while leaving viable cells unstained (Pierce, 1970). Since this method is widely reported to be inaccurate due to overestimation of live cells (O'Connor-Cox, *et al.*, 1997, Smart, *et al.*, 1999), the alternative fluorescent stains MgANS and oxonol were also employed. MgANS functions by entering non-viable cells and binding to cytoplasmic proteins in the form of highly fluorescence complex (McCaig, 1990). Similarly oxonol binds to intracellular proteins and lipids in cells when transmembrane potential is lost (Epps, *et al.*, 1994, Lloyd & Dinsdale, 2000). Despite the application of these three stains with different modes of action, they yielded broadly comparable data; the viability of each strain was observed to decrease with increased osmolality irrespective of the method used. This overall result was consistent with the findings of Panchal and Stewart (1980), who observed a decline in cell viability with increasing sorbitol-induced osmotic challenge when yeast was growing in shake flasks, particularly after the first five hours of growth. The data presented here therefore indicated that increasing external osmolality could be one of the primary causes of viability loss throughout fermentation. The data also suggests that it is likely that the reduction in viability can be directly apportioned to the external osmotic pressure and, as such, acts as a retrospective indicator of the impact of osmotic stress on cell physiology. In addition, it is suggested that osmo-tolerance may have a degree of strain-dependency, with the ale strain NCYC1332 appearing to be more osmo-sensitive than the other yeasts analysed in this study. Indeed, the sensitivity of brewing yeast cells in response to osmotic pressure has previously been suggested to be strain specific (Pratt, *et al.*, 2003, Yu, *et al.*, 2012) as discussed previously (Section 4.3.2).

Perhaps unsurprisingly, the data presented here also demonstrates that stationary phase cells of ale and lager yeast may be more tolerant to external osmotic pressure than their exponential counterparts. The difference in resistance is likely to be due to intrinsic physiological changes and altered expression levels of general stress response genes that are concomitant with the onset of stationary phase (Werner-Washburne, *et al.*, 1989, Panaretou & Piper, 1992, Werner-Washburne, *et al.*, 1993, Werner-Washburne, *et al.*, 1996). These modifications provide cells with the ability to survive for long periods of time during stressful conditions (Werner-Washburne, *et al.*, 1996, Ashrafi, *et al.*, 1999), and are likely to also aid cells against stress factors at later stage of fermentations. For instance, it is known that hyper-osmotic stress results in an increase of intracellular protein concentration and total protein density, primarily due to the activation of HOG pathway. Recently, Miermont, *et al.* (2013) demonstrated that increased osmotic compression could decrease protein mobility and slow down the dynamics of signalling cascades, such as the stress response pathways required for osmotic adaptation. Given that osmotic pressure has a negative effect on cell survival, it would be interesting to explore whether the delay of cellular signalling processes is a phase-dependent response, which may provide a possible explanation for the cell survival behaviour under different growth phases.

The fermentation performance of brewing yeast can be predicted using the acidification power test, a simple and sensitive method to assess the effect of process parameters on yeast vitality (Kara, *et al.*, 1988, Gabriel, *et al.*, 2008). In this assay, water acidification power (WAP) is believed to correlate with the metabolism of endogenous substrates such as glycogen and trehalose, whereas glucose acidification power (GAP) is related to the ability to utilize intra- and extra-cellular substrates (Siddique & Smart, 2000). Glucose induced proton efflux (GIPE) (calculated by subtracting the value of WAP from GAP; Section 2.11), was therefore used in this study, with the aim of obtaining the proton efflux resulting only from

glucose utilisation as a measure of cell vitality. Analysis of GIPE indicated that the capacity to utilize exogenous glucose was reduced with elevated extracellular osmolality for both of the strains studied, regardless of their growth phase. The data suggests that the increase in external osmolality may result in a reduced ability to pump protons out of the cell throughout fermentations. This has particular implications for HG and VHG brewing where elongated fermentations are frequently observed (Sigler, *et al.*, 2009, Yu, *et al.*, 2012). This finding is consistent with Yu, *et al.* (2011), who provided evidence that proton efflux rate is inhibited during the later stages of HG and VHG brewing fermentations. They also provided evidence that higher concentrations of ethanol had a significant inhibitory effect on proton efflux, although the authors did not link this to osmotic stress *per se*. Moreover, the ale strain NCYC1332 showed a lower activity than the lager strain W34/70 following exposure to 10 and 20 % (w/v) sorbitol, indicating that osmolality levels in the range of 563-1125 mOsm/kg had a more negative effect on the vitality of NCYC1332 compared to W34/70, further demonstrating the higher osmo-sensitivity observed in NCYC1332.

Analysis of intracellular trehalose revealed that this carbohydrate was initially consumed by both W34/70 and NCYC1332 yeast cells during starvation, as represented within the experimental conditions provided by 0 % (w/v) sorbitol. However, accumulation of trehalose was observed immediately following exposure to extracellular osmotic pressure, irrespective of cell growth phase. Interestingly, the final concentration of trehalose did not appear to be correlated to the level of osmolality, indicating that it may play an important role in protecting cells from damage even at very low levels of external osmotic pressure, or that it may be produced as part of a non-specific stress response. Moreover, accumulation of trehalose was more marked in stationary phase cells than in exponential phase cultures, consistent with the enhanced osmo-tolerance associated with stationary phase cells, as indicated during yeast viability analysis. It should be noted that despite the accumulation of

osmo-protectants, decreased cell viability with increasing osmolality was still observed. Indeed, Hounsa, *et al.* (1998) found that the hyper accumulation of trehalose in yeast mutants did not necessarily improve survival rates when compared to wild-type strains. Although trehalose has been regarded as a membrane stabilizer and stress protectant under stressful fermentations (Majara, *et al.*, 1996a, Majara, *et al.*, 1996b), it remains unclear to what extent this molecule acts to ‘rescue’ yeast cells, or the minimum and maximum effective concentrations required to protect individual cellular components.

Intracellular glycerol production was also evoked in both stationary and exponential phase populations following exposure to the osmolality levels applied. However, in this instance the production of glycerol appeared to be proportional to osmolality levels between 563 and 2813 mOsm/kg, indicating a relationship between the levels of external osmolality and the concentration of internal glycerol produced. Interestingly, the higher production of glycerol observed in strain W34/70 compared to NCYC1332 may in part explain the differences in osmotolerance between the two strains. Given that glycerol is primarily produced as a result of the activation of the HOG pathway, this result could be related to the expression levels of genes involved in the cellular signalling route, including *GPD1* and *GPP1*, involved in catalysing the conversion of dihydroxyacetonephosphate (DHAP) via glycerol-3-phosphate (G3P) to glycerol (Klipp, *et al.*, 2005). It is suggested that a systematic investigation of the relationship between external osmotic pressure and gene expression levels may be useful to fully elucidate this relationship in the future.

6.3.2 The impact of extracellular osmolality on membrane fluidity

A probe 1,6-diphenyl-1,3,5-hexatriene (DPH) has previously been applied in studies focused on the relationship between environmental parameters and membrane fluidity (Grunberger, *et al.*, 1982, Lentz, 1993, Laroche, *et al.*, 2001). However, it has been reported that the decay of

DPH shows little spectroscopic variation in the two phospholipid phases (Parasassi, *et al.*, 1991) and may not be suitable for quantitative measurements of membrane fluidity (Los & Murata, 2004). The alternative membrane probe employed here, laurdan (Weber & Farris, 1979), has the advantage of displaying spectral sensitivity to the phospholipid phase state (Parasassi, *et al.*, 1991), and has been shown to be an effective tool in the determination of membrane fluidity in response to heat and ethanol stress factors in yeast (Parasassi, *et al.*, 1991, Beney & Gervais, 2001, Learmonth & Gratton, 2002). Consequently this stain was adopted to examine the effect of extracellular osmotic pressure on membrane fluidity.

Data showed that the lipid bilayer of cells was composed of a mixture of liquid-crystalline and gel- phases, since the GP value was intermediate between the theoretical range from -1 to +1 (Parasassi & Gratton, 1995, Learmonth & Gratton, 2002). Although a possible increase in fluidity was observed when cells were exposed to low osmolality conditions, the predominant trend was that membrane fluidity gradually decreased with increasing osmotic pressure. Although cells of W34/70 and NCYC1332 were affected in slightly different ways, the membrane fluidity within populations was typically reduced to a similar degree under high osmolality conditions. This data confirmed the existence of a link between the membrane state and the extracellular osmolality, indicating that yeast cells are able to implement a change in membrane fluidity during growth in response to external osmotic pressure. The reduced membrane fluidity resulting from the modification of membrane structure and composition may act to compensate for changes in osmolality and to govern cell resistance to stress both directly and via cell signalling pathways (Beney & Gervais, 2001). Indeed, it was observed that stationary phase W34/70 cells displayed lower membrane fluidity than NCYC1332 in the presence of 10 and 20 % (w/v) sorbitol. Simultaneously, stationary phase cells of W34/70 also showed higher viability and vitality, as well as higher glycerol accumulation than their exponential phase counterparts. Interestingly, Kamada, *et al.* (1995)

proposed that a heat-induced signal for pathway activation is generated in response to a weakness in the cell wall, perhaps a consequence of decreased membrane fluidity. Moreover, the degree of lipid unsaturation and the presence of ergosterol in the membrane of *S. cerevisiae*, have been related to stress tolerance (Swan & Watson, 1999). Other studies have also demonstrated a relationship between the composition of membrane lipid and expression of stress-induced proteins (Chatterjee, *et al.*, 2000, Runner & Brewster, 2003, Rodriguez-Vargas, *et al.*, 2007). Nevertheless, it should be noted that membrane fluidity can be modified transiently or permanently by environmental stresses (Learmonth & Carlin, 1996, Learmonth & Carlin, 1997, Shah & Learmonth, 1998, Learmonth, 2011). In the case of extreme stress conditions, regulation may not compensate for the changes in the physical membrane characteristics, and may result in cellular damage or even death (Beney & Gervais, 2001, Learmonth, 2011). Thus a more detailed experimental analysis is required to further evaluate stress-induced membrane tolerance, membrane dynamics and structural characteristics, as well as the effect of process parameters on membrane properties in the future. Complementary techniques such as multi-photon scanning fluorescence microscopy could be applied as an additional dimension to the analysis, providing information on response of individual cells within population (Learmonth & Gratton, 2002, Learmonth, 2011).

6.3.3 The impact of extracellular osmolality on DNA integrity

Although genome instability, as a consequence of environmental stresses, has been suggested to provide stress adaptation in yeast strains, it may also have a negative effect on beer quality (Smart, 2007). In order to ascertain the potential impact of osmotic stress on brewing yeast DNA integrity, yeast inter-delta sequences were examined. Inter-delta sequences are DNA repeats that flank the Ty1 yeast retrotransposon and are known to be subject to frequent positional change within the genome (Ness, *et al.*, 1993). Consequently these elements are

good candidate targets for analysis of genetic variation and have been used previously to investigate potential genetic changes during serial repitching (Powell & Diacetis, 2007), as well as the analysis of the effect of dehydration and rehydration on active dried yeast genetic integrity (Jenkins, *et al.*, 2010).

An initial examination of inter-delta regions from yeast populations revealed strain-specific profiles for both yeast strains W34/70 and NCYC1332. However, analysis of DNA from cells subjected to extracellular osmotic pressure revealed that there were no detectable genetic changes within these regions, suggesting that cells were genetically stable over the range of osmolality tested (0 to 2813 mOsm/kg). Although this result is important, it was perhaps not entirely surprising, given that Powell and Diacetis (2007) demonstrated that no large scale genetic variation occurred during extended serial repitching of an ale strain (albeit at low gravity), and that Jenkins, *et al.* (2010) showed evidence to suggest that there was no variation in DNA profiles obtained from active dried yeast before and after dehydration and rehydration. However, it is acknowledged that there are significant differences between strains in terms of their propensity to form genomic mutants through successive generations (Adams, *et al.*, 1985, Adams, *et al.*, 1992, Smart & Whisker, 1996, Sato, *et al.*, 2001, Powell & Diacetis, 2007, Powell & Nguyen, 2012). Consequently, this data demonstrates that genomic DNA damage is not directly associated with the range of osmolality encountered within standard, HG and VHG brewery fermentations. However, it should be noted that mitochondrial DNA (mtDNA) may be a more susceptible site of damage, resulting in the formation of petite mutants (Goldring, *et al.*, 1970, Nagley & Linnane, 1970, Smart, 2007). Hence additional work on mtDNA sequence conformity would be needed to verify the genetic stability of the whole genome in response to osmotic stress.

6.4 Conclusion

In this chapter, fermenting wort media was investigated as a source of extracellular osmotic pressure, and the impact of this on yeast cell physiology was ascertained. Osmolality was observed to increase during fermentation with the largest contributor appearing to be derived from ethanol, with additional contributions from other compounds including wort and glycerol. These results highlighted the osmotic potential of ethanol during brewing fermentation, which has not previously been reported.

The effect of extracellular osmolality on cell physiology was investigated by the application of media containing various concentrations of sorbitol. Cell viability and vitality (proton efflux) were both negatively affected, although strain-dependent profiles were observed. Cells typically accumulated both trehalose and glycerol, however although glycerol production appeared to be directly correlated to the level of external osmolality, trehalose was not. Indeed, trehalose was observed to be produced even under low levels of stress, indicating that it may either act to protect cells subjected to minor stress, or that it may be produced as part of a non-specific stress response. It was also demonstrated that membrane fluidity decreases during growth on media designed to create a high external osmolality, and this can be due to changes in membrane structure, which may act to govern cellular resistance to osmotic stress both directly and via cell signalling pathways. Furthermore, analysis of DNA inter-delta regions indicated that cells were genetically stable irrespective of the degree of extracellular osmotic exposure applied. It is recognized that due to the nature of molecular based techniques, which require analysis of cell populations rather than individuals, DNA damage occurring at an extremely low rate may not be detected. However, at the basic genetic level there is no evidence to suggest that osmotic stress associated with high gravity brewing should lead to impaired performance as a result of DNA damage.

CHAPTER 7: CONCLUSION AND FUTURE WORK

7.1 Conclusion

In the brewing industry, traditional practice is to prepare wort to a standard gravity of approximately 11-12 °P leading to the production of fermented beer with 4-5 % (v/v) alcohol (Blieck, *et al.*, 2007). High Gravity (HG) or Very High Gravity (VHG) fermentations are procedures whereby worts of higher than normal concentration are used to produce high alcohol beer which consequently requires dilution at a later stage of the process. This practice has advantages, including enhanced productivity without significant capital expenditures on existing facilities, reduced energy, labour and cleaning cost, as well as the potential to increase product diversity by diluting a ‘mother beer’ to create a range of products (Stewart, 2009, Stewart, 2010, Puligundla, *et al.*, 2011). However, as the gravity of wort increases, the levels of biological stress factors also rise, which can have a profound impact on yeast quality and fermentation performance. Given that the conversion of wort sugars to alcohol is the primary function of yeast during fermentation, the impact of increased wort gravity on yeast central carbon flux and osmotic adaption is vital to a successful fermentation. The aim of this research was to address this issue by investigating the relationship between wort gravity, key fermentation performance indicators and yeast osmotic stress response under high gravity conditions. In this thesis, two lager and two ale brewing yeast strains were characterized (Chapter 3) and subsequently, a series of lab-scale fermentations were conducted using standard, HG and VHG worts (Chapter 4), leading to an estimation of yeast carbon partitioning affected by different wort densities (Chapter 5) and an investigation of yeast physiology in response to increasing osmotic pressure (Chapter 6).

Given that brewing yeast is the most vital component of beer production, and that strain phenotypic diversity can be both broad and narrow, characterization of yeasts is important to provide an insight into basic physiology prior to further analysis. In the current study (Chapter 3), two lager (Lager1 and W34/70) and two ale (NCYC1332 and M2) brewing yeast

strains were characterized according to their differences in permissive growth temperature and the potential to produce melibiase (α -galactosidase). These analyses were able to divide strains into their brewing classification (ale/lager), with lager strains unable to grow at 37 °C, but able to produce biomass on melibiose-based medium. Furthermore, each yeast strain was characterized via mtDNA RFLP fingerprinting. Although individual ale strains were identifiable based on mtDNA restriction profiles, lager strains yielded only a group-specific DNA fingerprint. However, subsequent physiological analysis demonstrated clear phenotypic differences between all of the strains demonstrating that each yeast was unique. Each yeast strain was assessed for the capacity to tolerate stress factors associated with HG and VHG brewing, including ethanol and osmotic stress. Increasing ethanol levels led to a gradual inhibition of yeast growth; whilst yeast strains were able to withstand ethanol within the range of 0-10 % (v/v), outside of this range ethanol was observed to significantly inhibit growth, ultimately leading to cell death. Yeast osmotic tolerance was tested by cultivation on wort-sorbitol plate, and by performing kinetic growth analysis using similar liquid media. Increasing sorbitol concentration (0-30 %, w/v) was shown to have a negative effect on yeast growth. This was particularly apparent for colony formation when concentrations above 18 % (w/v) were reached, and was further characterized by an extended lag phase and reduced maximum growth as the degree of osmotic pressure continued to increase. These preliminary examinations provided an overview of the phenotypic characteristics of each brewing strain selected to support further investigation into fermentation performance (Chapters 4-5) and yeast osmotic stress response (Chapter 6) under HG and VH conditions.

Previous studies into the relationship between wort gravity and fermentation performance (Younis & Stewart, 1999, Saerens, *et al.*, 2008, Sigler, *et al.*, 2009, Dekoninck, *et al.*, 2012, Lei, *et al.*, 2012, Yu, *et al.*, 2012) have suggested that increasing wort density may greatly affect yeast carbon metabolism. This is particularly important since carbohydrates are the

most abundant wort nutrient, especially under HG and VHG brewing, and the majority of fermentation products are also carbon-based. In this study (Chapter 4), a series of lab-scale fermentations were conducted using 13 °P, 18 °P and 24 °P commercial worts. For each set of fermentations, pitching rate and oxygen conditions were adjusted to examine their combined effects on yeast fermentation and key performance indicators. With a ‘standard’ pitching rate (1.5×10^7 viable cells/mL, irrespective of initial wort Plato), the increased wort gravity resulted in an accelerated viability loss, a slower fermentation rate and incomplete sugar utilisation. Production of ethanol, carbon dioxide and glycerol occurred at a concentration broadly proportional to the original wort gravity, whereas the maximum glycogen produced was not affected by wort density, and the accumulation of trehalose was strain-specific. When comparing an adjusted pitching rate (1.0×10^6 viable cells/mL per degree Plato) to the ‘standard’ pitching rate at high gravities (18 °P and 24 °P), the data suggested that the former could not only function to accelerate the fermentation process, but may also result in a more complete carbohydrate utilisation, leading to an enhanced ethanol yield without significant alterations to flavour biosynthesis. The adjusted pitching rate described above was calculated based on the generally regarded ‘rule of thumb’ in brewing practice, and the results indicate the benefits of making adjustments centred around this principle for VHG fermentations. It should be noted that, whilst the inoculum preparation method used in this study was different to that in general brewing practice (aerobic versus anaerobic yeast growth condition prior to pitch, as discussed in Section 4.3.1), the potential impact on yeast physiology and fermentation performance should be identical in each condition. It is proposed that the specific pitching rate should be further optimised for individual brewing strains at high gravity, allowing the brewer to control the extent of cell growth and ultimately leading to optimisation of fermentation efficiency.

Management of ethanol production from wort carbohydrates is particularly important for HG and VHG brewing fermentations in order to maximise fermentation efficiency. However, the use of highly concentrated worts is concomitant with a number of biological stress factors which can affect yeast carbon flux. Therefore, the way in which brewing yeast adapt their central carbon partitioning in response to the wort environment is critical in determining both fermentation efficiency and the health of the yeast culture; an important consideration if the yeast culture is to be re-pitched. Based on the fermentation analysis conducted previously (Chapter 4), an approach was described to evaluate the effect of sugar concentrations on yeast carbon partitioning during fermentations (Chapter 5). The estimation of total carbon input was determined from the carbohydrate composition of the original wort and final beers, and the carbon partition in each metabolite was expressed as a percentage of the total carbon input. Data revealed that, although produced to higher concentrations overall during high gravity fermentations, the majority of carbon-based products, including trehalose, glycogen, higher alcohols and esters had only minor effects on the percentage of carbon distribution. It was observed that yeast biomass production acted as the major trade-off with ethanol yield, reinforcing the suggestion that the control of cell growth is potentially the most important strategy to optimise carbon into ethanol production, as described above.

Current understanding within the industry is that the use of HG and VHG worts results in increased osmotic stress at the beginning of fermentation due to the high concentration of sugars present (Stewart, 2010, Puligundla, *et al.*, 2011), after which osmotic stress becomes gradually reduced. However, the precise changes of osmotic pressure in fermenting media, as well as the impact of this on brewing yeast physiology related to VHG brewing, have not been fully explored. To address this, osmolality (as a measure of osmotic pressure expressed in mOsm/kg), was used to profile the range of external osmotic pressures encountered during fermentations (Chapter 6). In contrast to previous belief, osmolality was observed to increase

during fermentations, with the largest contribution derived from ethanol production, along with lesser inputs from other compounds including wort and glycerol. These results highlighted the osmotic potential associated with ethanol during brewing fermentations, particularly at high gravities, but also when using standard worts, indicating that the role of different yeast stress factors may be interlinked to a great extent than previously considered. Consequently, a series of environments designed to mimic the range of osmolality levels encountered by cells during fermentations were created using the sugar-alcohol molecule sorbitol, and the effect of external osmolality on cell physiology was investigated. Cell viability and vitality (proton efflux) were both observed to be negatively affected by increased osmotic pressure, although strain-dependent profiles were observed indicating variation in effect between yeasts. In response to osmotic pressure, trehalose and glycerol accumulation was also observed and, interestingly, trehalose was observed to be produced even under low levels of pressure, while glycerol production was more directly related to osmolality. It was also demonstrated that cells respond to increased external osmolality by actively decreasing membrane fluidity, most likely resulting from changes in membrane structure. It was suggested that this could act to initiate osmo-regulation both directly and via cell signalling pathways (Beney & Gervais, 2001). Furthermore, in contrast to previous studies involving successive generations of brewing yeast (Adams, et al., 1985, Adams, et al., 1992, Smart & Whisker, 1996, Sato, et al., 2001, Powell & Diacetis, 2007, Powell & Nguyen, 2012), analysis of DNA interdelta regions indicated that cells were genetically stable irrespective of the degree of extracellular osmotic pressure applied. This indicates that the production strains investigated here may be genetically robust, at least within the range of osmolality levels typically encountered within the brewing process.

In summary, this work provides evidence to suggest that, for the current strains investigated, significant time savings to the VHG fermentation process and enhanced ethanol yield in the

final product are most likely to be influenced by pitching rate. A carbon trade-off between yeast biomass and ethanol was observed, demonstrating that maximisation of ethanol yield could be achieved by control of cell growth during fermentation. However, since other carbon end products such as trehalose and glycerol did not have a significant impact on ethanol yield, it is possible that the use of alternative strains, which inherently display a preference for the production of these compounds, may actually prove to more appropriate for the creation of high ethanol products in the future. Such strains may divert a greater proportion of carbon towards cellular protective agents at the expense of ethanol production, but this may ultimately lead to improved yeast physiology in general and shorter fermentation times: two key parameters in defining fermentation efficiency. It should also be noted that current strains have been selected over many years for their favourable properties at relatively low gravity and it is only in recent years that brewers have seriously considered exploiting HG or VHГ fermentations for high volume lager-style products. This practice has changed expectations of the current yeast strains employed, and it may be that alternative organisms, comprising a more favourable phenotype overall, should be considered for the purpose of HG and VHГ brewing. Consequently, it is anticipated that the data presented here will lead to a greater understanding of the phenotypic requirements of yeast for HG and VHГ brewing, leading to process optimisation in the future. Furthermore, the significant contribution of ethanol to osmolality observed in this study provides a springboard for future work on the implication of protracted osmotic pressure for both brewery and other yeast-based fermentation systems (e.g. wine, biofuels).

7.2 Future work

There are several areas of research which have the potential to both complement and expand on the data presented here:

(I) Carbon metabolic flux analysis using tracer-based techniques: although an approach to calculate carbon partitioning during brewing fermentation has been described in this research programme, this was developed based on the observations of Cutaia (2007). In order to remove potential errors caused by the ‘assumption’ of biomass, carbon labelling techniques using nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (Cai, *et al.*, 2011) could be employed to provide a more precise quantification. Given the complex sugar composition in the wort media, this analysis may begin with analysis of single wort carbohydrate labelled with isotopes ^{13}C before cultivation on multiple carbon sources. Furthermore, the intracellular carbon flow during fermentation could also be obtained through ^{13}C -based metabolic analysis, and the carbon flux to important intermediates such as pyruvate and acetyl-CoA could be monitored. This would assist in the analysis of yeast strains with regard to the search for new metabolic engineering strategies to manage ethanol content in batch fermentations.

(II) Optimisation of cell growth for maximisation of ethanol yield: the work performed in this study indicates a carbon trade-off between biomass and ethanol during HG and VHG brewing fermentations. However, the precise dynamics for optimizing cell growth and maximizing ethanol yield remain to be investigated. Pitching rate and wort dissolved oxygen (DO) concentration are particularly important as the former provides the initial cell numbers for fermentation and the latter plays a major role in lipid biosynthesis (Rattray, *et al.*, 1975, Rosenfeld, *et al.*, 2003), essential for yeast growth. As a general rule, it is suggested to pitch 1 million viable cells/mL per degree Plato and to oxygenate at 1 ppm oxygen per degree Plato prior to fermentation (Stewart, 2009). However, these practices have largely been employed for fermentations at relatively low gravity and have not been tested sufficiently for high gravity worts. Consequently it is hypothesised that a series of experiments focused on optimising initial cell density and oxygenation for individual strains in VHG worts should be

performed to calculate the efficient balance between biomass and ethanol production (Boulton & Quain, 1985).

(III) The effect of process conditions on the profile of membrane fluidity during brewing fermentation: the present study was restricted to evaluating the impact of artificially induced osmotic pressure on membrane fluidity, and the direct impact of fermentation on the cell membrane was not investigated. While the current study provides valuable insight, it is important to further investigate changes in membrane fluidity during fermentation process, taking into consideration the influence of process parameters such as oxygen and temperature. As described previously, oxygen is required for the biosynthesis of lipids, including the conversion of squalene into ergosterol and the formation of double bonds in unsaturated fatty acids (UFAs) (Ratnayake, *et al.*, 1975, Rosenfeld, *et al.*, 2003). It is hypothesised that the initial wort DO levels may play an important role in the biophysical characteristics of membrane fluidity which may in turn impact on the degree to which a cell can respond to environmental changes. In addition, membrane fluidity can be a function of temperature (Laroche, *et al.*, 2001, Simonin, *et al.*, 2008); typically lager beers are fermented at 8-15 °C whereas ale beers are produced at around 20 °C (Walsh & Martin, 1977, Boulton & Quain, 2001). It would be interesting to determine the effect of temperature on the profile of brewing yeast membrane fluidity. This could lead to a better understanding of factors determining membrane structure and provide insight into the potential for manipulating such parameters for the production of high gravity beers in the future.

(IV) The effect of external osmotic pressure on stress-responsive genes using quantitative real-time PCR: the present work examined the osmotic stress response of brewing yeast with respect to cellular function and integrity. However, it would be useful to reinforce this with a study of the stress response at the molecular level. Of particular importance are the stress

responsive element (STRE)-driven genes including *HSP104*, *TPS1*, *TPS2*, *TPS3*, *TSL1*, *NTH1* and *NTH2* (Winderickx, *et al.*, 1996, Zahringer, *et al.*, 2000), as well as the target genes in High Osmolarity Glycerol (HOG) osmo-sensing pathway such as *HOG1* (Brewster, *et al.*, 1993), *PBS2* (Boguslawski, 1992) and *GPD1* (Albertyn, *et al.*, 1994). The expression levels of these genes and the concentrations of the corresponding cellular compounds could lead to a better understanding of the stress response under HG and VHG conditions, allowing the effect of individual components of the fermentation media to be more precisely characterised. Furthermore, the osmo-tolerant yeasts (Section 1.6.3) may provide a benchmark to the behaviour of *Saccharomyces* yeasts in high gravity fermentations.

(V) The effect of wort gravity on intracellular osmolality during brewing fermentations: the current study revealed an increased extracellular osmotic pressure during fermentations; however this was restricted to the evaluation of the level of osmotic imbalance that the whole yeast cells encountered, since intracellular osmolality was not considered. It is believed that *S. cerevisiae* is able to monitor internal osmotic pressure in order to maintain a viable and optimal volume (Meikle, *et al.*, 1988) and the ability of cells to sense and respond to a change in external osmolality is therefore fundamental for yeast growth and survival (Runner & Brewster, 2003). In the future it would be interesting to perform a study which simultaneously investigates the change in internal osmolality, and to determine the level of osmotic imbalance between the internal and external osmotic pressure during fermentation. This would allow a more complete picture of the relationship between osmolality, fermentation and yeast physiology to be determined. Additionally, internal compounds other than glycerol (such as trehalose and glycogen) may provide a contribution to the intracellular osmolality, as well as other components including amino acids, ions and sulphonium compounds. Understanding the mechanisms of this regulation could provide a possible means of monitoring and explaining the performance of yeast in the complex brewing environment.

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